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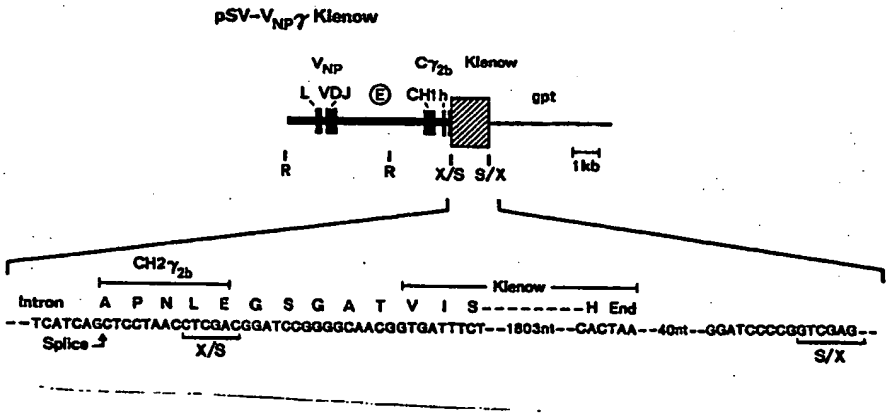
(54) Title: PRODUCTION OF CHIMERIC ANTIBODIES



(57) Abstract

A process for the production of a chimeric antibody, comprising: a) preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence comprising a first part which encodes at least the variable region of the heavy or light chain of an Ig molecule and a second part which encodes at least part of a second protein; b) if necessary, preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable region of a complementary light or heavy chain respectively of an Ig molecule; c) transforming an immortalised mammalian cell line with the or both prepared vectors; and d) culturing said transformed cell line to produce the chimeric antibody; chimeric antibodies produced by this process; and plasmids and transformed cell lines used in the process.

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PRODUCTION OF CHIMERIC ANTIBODIES

The present invention relates to a process for the production of chimeric antibodies using recombinant DNA techniques.

In the present application, the term 'chimeric antibody' is used to describe a protein comprising at least the antigen binding portion of an immunoglobulin molecule (Ig) attached by peptide linkage to at least part of another protein.

In recent years, advances in molecular biology based on recombinant DNA techniques have provided processes for the production of a wide range of heterologous polypeptides by transformation of host cells with heterologous DNA sequences which code for the production of the desired products.

EP-A-O 088 994 (Schering Corporation) proposed the construction of recombinant DNA vectors comprising a ds DNA sequence which codes for a variable region of a light or a heavy chain of an Ig specific for a predetermined ligand. The ds DNA sequence is provided with initiation and termination codons at its 5'- and 3'- termini respectively, but lacks any nucleotides coding for amino acids superfluous to the variable region. The ds DNA sequence is used to transform bacterial cells. The application does not contemplate the production of chimeric antibodies.

EP-A-1 102 634 (Takeda Chemical Industries Limited) describes the cloning and expression in bacterial host organisms of genes coding for the whole or a part of human IgE heavy chain polypeptide, but does not contemplate the production of chimeric antibodies.

EP-A- 0 125 023 (Genentech Inc. et al.), which was published after the priority date of the present

application, proposes the use of recombinant DNA techniques in bacterial cells to produce Ig's which are analogous to those normally found in vertebrate systems and to take advantage of the gene modification techniques proposed therein to construct chimeric Ig's or other modified form of Ig.

It is believed that the proposals set out in the above Genentech application did not lead to the expression of any significant quantities of Ig polypeptide chains, nor to the production of Ig activity, nor to the secretion and assembly of the chains into the desired chimeric Ig's.

The production of monoclonal antibodies was first disclosed by Kohler and Milstein (Kohler, G. and Milstein, C., Nature, 256, 495-497, 1975). Such monoclonal antibodies have found widespread used not only as diagnostic reagents (see, for example, 'Immunology for the 80s, Eds. Voller, A., Bartlett, A., and Bidwell, D., MTP Press, Lancaster, 1981) but also in therapy (see, for example, Ritz, J. and Schlossman, S.F., Blood, 59, 1-11, 1982).

The recent emergence of techniques allowing the stable introduction of Ig gene DNA into myeloma cells (see, for example, Oi, V.T., Morrison, S.L., Herzenberg, L.A. and Berg, P., PNAS USA, 80, 825-829, 1983; Neuberger, M.S., EMBO J., 2, 1373-1378, 1983; and Ochi, T., Hawley, R.G., Hawley, T., Schulman, M.J., Traunecker, A., Kohler, G. and Hozumi, N., PNAS USA, 80, 6351-6355, 1983), has opened up the possibility of using in vitro mutagenesis and DNA transfection to construct recombinant Ig's possessing novel properties.

However, it is known that the function of an Ig molecule is dependent on its three dimensional structure, which in turn is dependent on its primary amino acid sequence. Thus, changing the amino acid sequence of an Ig may adversely affect its activity. Moreover, a change in the DNA sequence coding for the Ig may affect the ability of the cell containing the DNA sequence to express, secrete or assemble the Ig.

It is therefore not at all clear that it will be possible to produce functional altered antibodies by recombinant DNA techniques.

Similar considerations apply to other proteins. It therefore cannot be expected that fusion of a gene coding for at least part of an Ig with a gene coding for at least part of another protein will lead expression of any protein, let alone expression of protein which can be secreted and assembled to give a functional chimeric antibody.

However, the present inventors have now discovered unexpectedly that it is possible to produce by recombinant DNA techniques secreted, assembled chimeric antibodies in which both parts of the protein are functional.

This surprising result is achieved by the use of the process of the present invention, which comprises:

a) preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence comprising a first part which encodes at least the variable region of the heavy or light chain of an Ig molecule and a second part which encodes at least part of a second protein;

b) if necessary, preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable region of a complementary light or heavy chain respectively of an Ig molecule;

c) transforming an immortalised mammalian cell line with the or both prepared vectors; and

d) culturing said transformed cell line to produce a chimeric antibody.

The immortalised cell line is preferably of lymphoid origin, such as a myeloma, hybridoma, trioma or quadroma cell line. The cell line may also comprise a normal lymphoid cell, such as a B-cell, which has been immortalised by transformation with a virus, such as the Epstein-Barr virus. Most preferably, the immortalised cell line is a myeloma cell line or a derivative thereof.

It is known that some immortalised lymphoid cell lines, such as myeloma cell lines, in their normal state secrete isolated Ig light or heavy chains. If such a cell line is transformed with the vector prepared in step a) of the process of the invention, it will not be necessary to carry out step b) of the process, provided that the normally secreted chain is complementary to the part of the Ig molecule encoded by the first part of the vector prepared in step a).

However, where the immortalised cell line does not secrete or does not secrete a complementary chain, it will be necessary to carry out step b). This step may be carried out by further manipulating the vector produced in step a) so that this vector encodes not only the fusion of variable region and

second protein, but also the complementary variable region. However, preferably step b) is carried out by preparing a second vector which is used to transform the immortalised cell line.

The techniques by which such vectors can be produced and used to transform the immortalised cell lines are well known in the art, and do not form any part of the invention. However, they are well illustrated in the following Examples.

In the case where the immortalised cell line secretes a complementary light or heavy chain, the transformed cell line may be produced by transforming a suitable bacterial cell with the vector and then fusing the bacterial cell with the immortalised cell line, e.g. by Spheroplast fusion.

The first part of the DNA sequence may be joined directly to the second part thereof. Alternatively, the first part may be joined to the second part by an intervening sequence which encodes a specific cleavage sequence, for instance a Factor Xa cleavage sequence as described in our copending European patent application No. 85303414.8. Reference may be made to this application for further discussion of the use to specific cleavage sequences.

The second part of the DNA sequence may encode:

i) at least part, for instance the constant region of a heavy chain, of an Ig molecule of different species, class or subclass;

ii) at least the active portion or all of an enzyme;

iii) a protein having a known binding specificity;

iv) a protein expressed by a known gene but whose sequence, function or antigenicity is not known; or

v) a protein toxin, such as ricin.

The chimeric antibody produced in case i) above will be of use in a number of applications. For instance, an established cell line may produce an Ig molecule having a useful specificity. However it may be of a class which is diagnostically or therapeutically undesirable, or it may not be secreted in useful quantities. For instance, Ig of class M is known to be difficult to use in rapid immunoassay techniques and is generally inconvenient for use in therapy, whereas Ig of class G can be readily used in these techniques. Therefore, it would be possible to produce a useful immunoassay reagent or therapeutic agent by replacing the IgM heavy chain constant region with an IgG heavy chain constant region. A particular example of such use would be in the production of an chimeric antibody having an anti Rh specificity, derived from an IgM secreting myeloma and IgG reactivity provided by an IgG derived heavy chain constant region.

Alternatively, the chimeric antibody could comprise an IgG derived variable region and an Ig E derived heavy chain constant region. Such a chimeric antibody could be used to investigate the action of IgE on mast cells, in diagnostic assays, for instance calibrating test procedures or, in therapy, to inhibit allergic reactions caused by the action of normal IgE molecules on mast cells.

In another alternative, the chimeric antibody may be used to alter the complement binding activity of an antibody, again by changing the heavy chain constant region.

In a further alternative, the chimeric

antibody may be constructed to resemble an $(Fab')_2$ fragment of a normal antibody.

The chimeric antibody produced in case ii) above may be used, in particular, in an enzyme linked immunoassay (ELISA) system, in place of the present use of separate antibodies and enzymes.

The chimeric antibody produced in case iii) above may also be used in immunoassays. For instance, the protein may have a binding specificity for an easily detectable label, such as a heavy or radioactive metal or a dyed or dyeable molecule. For instance, it would be possible to produce a divalent chimeric antibody having a different variable region at each end, the ends being connected by at least a part of a constant region.

The chimeric antibody produced in case iv) above may be used to investigate the products of known genes. For instance, it may be known that a particular gene produces a protein involved in the surface marking of a cell. However, the exact nature of the surface marker may not be known. The chimeric antibody produced in case iv) will comprise the protein product of the gene, which can therefore be more readily characterised. Moreover, antibodies to this gene product could be raised and used to investigate with certainty the distribution of the gene product on a cell surface.

The chimeric antibody produced in case v) above will clearly be of use in therapy, for instance as a targetted cytotoxic agent for cancer therapy. In this respect reference may be made to an article by Thorpe et al. (Thorpe, P.E., Edwards, D.C., Davies, A.J.S. and Ross, W.C.J., in 'Monoclonal Antibodies in Clinical Medicine', 167-201, Eds.,

McMichael, A.J. and Fabre, J.W., Academic Press, 1982).

The chimeric antibodies produced by the present process, especially where the DNA sequence encodes a specific cleavage site, may be used for purifying the second protein. For instance, the variable region may be made specific for a hapten which can be immobilised on a chromatography medium. The chimeric antibody can then be immobilised by affinity chromatography and contaminating material can be washed away. The second protein can then be cleaved from the variable region either before or after the variable region is eluted from the chromatography medium.

The chimeric proteins of the type referred to in cases ii) to iv) above and in the preceding paragraph also comprise aspects of the present invention.

The present invention is described in more detail, by way of non-limiting illustration only, with reference to the accompanying drawings; in which:

Figure 1A shows the structure of plasmid pSV-Vul;

Figure 1B shows the predicted structure of the IgM molecule produced by the J558L cell line transformed with the plasmid pSV-Vul;

Figure 2 shows polyacrylamide gels of the purified IgM molecule produced by the J558L cell line transformed with the plasmid pSV-Vul;

Figure 3A shows the structure to plasmid pSV-$V_{NP}\gamma\delta$;

Figure 3B shows the predicted structure of the F(ab)'$_2$ chimeric antibody produced by the J558L cell line transformed with the plasmid pSV-$V_{NP}\gamma\delta$;

Figure 4 shows polyacrylamide gels of purified products produced by the J558L cell line transformed with various plasmids;

Figure 5 shows polyacrylamide gels of purified products produced by the J558L cell line transformed with various plasmids in the presence or absence of tunicamycin;

Figure 6A shows the structure of plasmid pSV-V_{NP} γSNase;

Figure 6B shows the predicted structure of the chimeric antibody produced by the J558L cell line transformed with the plasmid pSV-V_{NP} γ SNase;

Figure 7 shows polyacrylamide gels used for monitoring SNase activity;

Figure 8 shows an EISA-type assay;

Figure 9 shows the structure of plasmid pSV-V_{NP} γ myc;

Figure 10 shows an assay for c-myc antigenic determinant in Fab-myc;

Figure 11 shows the structure of plasmid pSV-V_{NP} H ;

Figure 12 shows a serological analysis of chimeric IgE by binding inhibition assays;

Figure 13 shows polyacrylamide gels of purified chimeric IgE;

Figure 14A shows the structure of plasmid pSV-V_{NP} γ Klenow;

Figure 14B shows the predicted structure of the Fab-γ Klenow chimeric antibody produced by the J558L cell line transformed with plasmid pSV-V_{NP} γ-Klenow;

Figure 15 shows polyacrylamide gels of the purified Fab-γ Klenow chimeric antibody; and

Figure 16 shows an assay for Klenow fragment activity.

In the drawings which show plasmids, thin horizontal lines depict pSV2 gpt vector, thick lines represent mouse or human Ig gene DNA, exons are represented by boxes, and hatched areas represent second (non-Ig) protein DNA.

The locations of the heavy chain locus transcription enhancer element (E) and the gpt gene are indicated. Restriction endonuclease cleavage sites are abbreviated as follows:
R = Eco RI; Xh = Xho I; S = Sac I; and Sl/Xh = a sequence formed by joining a Sal I site to an Xho I site. The sequence is presented around the Xho I site of plasmid pSV-V_{NP} γSNase, which forms a junction of the γ 2b CH2 exon and the SNase gene.

In the diagrammatic representations of the predicted structures of Ig molecules and chimeric antibodies, disulphide linkages between heavy (H) and light (λ) chains are indicated by (-S-). Only one subunit of the decavalent pSV-Vul encoded IgM is illustrated.

In the following Examples use is made of an established mouse plasmacytoma cell line J558L which secretes λ_1 light chains but does not produce any Ig heavy chain. This plasmacytoma cell line is described by Oi et al. (Oi, V.T., Morrison, S.L., Herzenberg, L.A., and Berg, P., PNAS USA, 80, 825-829,1983).

In the Examples, reduced samples were analysed on 12% polyacrylamide gels, while unreduced samples were analysed on 7% polyacrylamide gels.

The Examples also use plasmid pSV-Vμ1 described by Neuberger, M.S., EMBO J., 2, 1373-1378, 1983. This plasmid comprises a complete mouse immunoglobin μ gene cloned into the expression vector pSV2 gpt, and is shown in Figure 1A. The Ig μ polypeptide encoded by this plasmid has a heavy chain variable region, V_H, characteristic of λ_1 light chain-bearing mouse antibodies which are specific for the hapten 4-hydroxy-3-nitrophenacetyl (NP): NP binding activity should therefore be formed following association of the pSV-Vμ1 encoded heavy chain with mouse λ_1 light chains.

To confirm this, pSV-Vμ1 DNA was introduced by spheroplast fusion into the J558L cell line and growth in selective medium essentially as described in the Neuberger article referred to above, except that HAT was omitted from the selective medium and mycophenolic acid was used at 5μg/ml, as described in the Oi et al. article referred to above. Cells were cloned by limiting dilution. Antibody samples were purified from supernatants of cloned J558L transfectants grown in Dulbecco's modified Eagle's medium containing 5% foetal calf serum. Supernatants (2 litres) were passed over 2ml columns of 4-hydroxy-5-iodo-3-nitrophenacetyl-amino-caproic acid Sepharose (NIPcap-Sepharose) and antibody eluted from the washed sorbent with 1mM NIPcapOH in phosphate buffered saline.

Biosynthetically labelled antibody was purified on 40 µl NIPcap-Sepharose columns from supernatants of cells incubated for 4h at 37°C in medium containing L-[^{35}S]-methionine. Tunicamycin was, if required, included during the labelling and during a 2h preincubation at 8µg/ml (parallel incubations with an IgE secreting cell line confirmed the efficacy of this treatment).

Stably transfected cells were selected and cloned by limiting dilution. Twenty clones were analysed by radioimmunoassay and each secreted high levels of an NP-specific IgM antibody. As illustrated in Figure 2, homogeneous anti-NP IgM antibody can be purified from supernatants of pSV-Vµl transfected clones with a yield of about 3mg/ml.

In Figure 2 : lane (a) shows purified antibody (30µg) which has been boiled with 2-mercaptoethanol prior to electrophoresis and stained with Coomassie blue; and lane (b) shows markers to enable the molecular weight of the chains to be estimated. The predicted structure of the Ig molecule isolated from pV-Vµl transfected clones is shown in Figure 1B.

Example 1 - F(ab)'$_2$ - like chimeric antibody

A derivative of pSV-Vµl was constructed in which the Cµ exons were replaced by the CH1 and hinge exons of the mouse γ2b gene. To provide translation termination and polyadenylation sequences, an exon, Cδs, derived from the gene encoding secreted mouse δ chains was placed at the 3' end of the gene. The constructed plasmid pSV-V_{NP}γδ is shown in Figure 3A.

To construct plasmid pSV-V_{NP}γδ, the V_{NP} exons from pSV-Vµl contained on a common Eco RI fragment are placed in a vector consisting of the Bam HI - Eco RI fragment of pSV2gpt (see Mulligan, R.C. and Berg, P., PNAS USA, 78, 2072-2076, 1981) with an Xho I adapter in the Bam HI

site. The plasmid contains an Eco RI - Sac I mouse Cγ2b fragment derived from phage λMYG9 (see Neuberger, M.S. and Calabi, F., Nature, 305, 240-243, 1983). The mouse $C\delta_s$ exon as the PSV-$V_{NP}\gamma\delta$ plasmid is contained in a Bam HI fragment of phage Ch 257 3 (see Cheng, H. - L., Blattner, F. R., Fitzmaurice, L., Mushinski, J.F., and Tucker, P.W., Nature, 296, 410-415, 1982) which was obtained as a Sac I - Sal I fragment after cloning in M13mp 11.

The truncated heavy chain gene of plasmid pSV-$V_{NP}\gamma\delta$ would be expected to direct the synthesis of a $F(ab)'_2$ - like chimeric antibody, as shown diagrammatically in Figure 3B, consisting of two IgG2b Fab molecules disulphide linked together through the γ2b hinge with a 21 amino acid tail piece at the carboxy terminus encoded by the $C\delta_s$ exon.

Plasmid pSV-$V_{NP}\gamma\delta$ was transfected into J558L cells and radioimmunoassay revealed that stably transfected cells secreted high levels of λ_1-bearing anti-NP antibody. This NP-specific antibody was purified from culture supernatants of several transfected clones with a yield of 5 to 10mg/l. Polyacrylamide gel electrophoresis of the purified material (Figure 4) shows that the major protein species has an unreduced molecular weight of about 110,000 daltons. After reduction, a band comigrating with λ light chain as well as several higher molecular weight polypeptides are observed. The most abundant of these larger polypeptides has a molecular weight of 31,000 and would constitute the heavy chain of the $F(ab)'_2$ like antibody. However, there is clear contamination of the pSV-$V_{NP}\gamma\delta$ $F(ab)'_2$ antibody with other NP-binding material that has an unreduced molecular weight of around 50,000 daltons and is composed of λ_1 light chains and one of several heavy chains in the molecular weight range 36,000 to 40,000. The presence

of this minor antibody component does not reflect glycosylation heterogeneity as the electrophoretic mobility of the pSV-$V_{NP}\gamma\delta$ encoded anti-NP antibody is unaffected by inclusion of tunicamycin in the incubation medium during biosynthetic labelling experiments (Figure 5). It is likely that the minor bands differ from the F(ab)'$_2$ antibody in the carboxy terminal portion of the heavy chain, possibly as a result of alternative processing of pSV-$V_{NP}\gamma\delta$ immunoglobulin gene RNA transcripts. Nevertheless, despite the contaminating bands, it is clear that F(ab)'$_2$-like anti-NP antibody can be synthesized and secreted in good yield by pSV-$V_{NP}\gamma\delta$ transfected J558L cells.

Example 2- Fab-nuclease chimeric antibody

A DNA restriction fragment containing the S. aureus nuclease (SNase) gene was inserted into the Xho I site located in the CH2 exon of the mouse γ2b gene. Plasmid pSV-V_{NP} SNase was assembled by inserting the V_{NP} exons contained on the common Eco RI fragment into the vector comprising the Bam HI - Eco RI fragment of pSV2gpt with an Xho I adapter in the Bam HI site as described in Example 1. An Eco RI - Xho I mouse Cγ2b fragment derived from phage λMYG9 was also inserted in the vector, so that pSV-$V_{NP}\gamma$SNase contains the γ2b CH1, hinge and 5' end of the CH2 exons. The SNase coding region is derived from an M13mp8 clone containing an S. aureus Sau 3A fragment in the Bam HI site. Removal of the SNase gene from M13mp8 as a Bam - Sal I fragment and recloning in M13mp12W (see Karn, J., Mathes, H. W. D., Gait, M. J., and Brenner, S., Gene, 32, 217-224, 1984) allowed its isolation as an Xho I - Sal I fragment for final assembly of the pSV-V_{NP} SNase, the structure of which is shown in Figure 6A.

The heavy chain gene of pSV-$V_{NP}\gamma$SNase is similar to that of pSV-$V_{NP}\gamma\delta$ except that the $C\delta_S$ exon has been removed and replaced by an exon containing the first four

codons of the γ2b CH2 exon fused in phase to the nuclease coding region. SV40-derived sequences of the pSV2gpt-derived vector provide polyadenylation signals.

J558L cells were transfected with pSV-V_{NP}γSNase and cells surviving in selective medium were cloned by limiting dilution. Radioimmunoassay of supernatants of cloned transfectants revealed that about one third were positive for the production of λ-bearing anti-NP antibody. Positive clones yielded between 1 mg/l and 10 mg/l of NP binding antibody, which has the predicted structure shown in Figure 6B.

Analysis of biosynthetically labelled antibody by gel electrophoresis reveals a band comigrating with λ_1 light chain as well as two heavy chain bands of molecular weight 45,000 and 46,000 (Figure 5). The difference between these two heavy chains has not been identified but their mobilities agree well with the predicted mobility of the V_{NP}γSNase heavy chain. Although the sequence Asn-Asn-Thr is present in SNase, the two V_{NP}γSNase heavy chain bands are still present in samples purified from supernatants of cells that have been biosynthetically labelled in the presence of tunicamycin (Figure 5). This demonstrates that the difference between the two pSV-V_{NP}γSNase heavy chains is not due to N-linked glycosylation. The V_{NP}γSNase antibody appears somewhat more heterogenous on a non-reduced gel, giving bands with the expected mobilities of both the $F(ab)'_2$-SNase and Fab-SNase (Figure 4). The presence of SNase on the heavy chain carboxy terminus might inhibit disulphide linking of the γ2b hinge regions.

To test for nuclease activity in the V_{NP}γSNase preparation, samples which had been purified on hapten-Sepharose columns were incubated with single stranded DNA substrate. Digestion of the DNA was monitored following agarose gel electrophoresis as

follows. Single stranded M13DNA (2µg) was incubated at 37°C for 30 minutes in 25 mM sodium borate, 250 mM NaCl, 10mM $CaCl_2$, PH 8·5 (20µl) containing varying amounts of $V_{NP}\gamma\delta$ SNase chimeric antibody or of purified S. aureus nuclease. The quantities of antibody/enzyme used are given in nanograms. DNA in the samples was then analysed by ethidium bromide fluorescence after electrophoresis through a 1·2% agarose gel. A Hind III digest of phage DNA provides size markers. Ca^{++} dependency of the nuclease activity was confirmed by running incubations in the presence of 40mM $MgCl_2$, 25mM EGTA.

As shown in Figure 7, $V_{NP}\gamma\delta$ SNase but not $V_{NP}\gamma\delta$ antibodies show nuclease activity and this activity - like that of authentic S. aureus nuclease - is dependent on Ca^{++} but not Mg^{++} ions. As judged on a molar basis, the catalytic activity of the $V_{NP}\gamma\delta$ SNase sample is about 10% that of authentic S.aureus nuclease.

The $V_{NP}\gamma\delta$ SNase chimeric antibody can be used as a genetically conjugated enzyme linked antibody in ELISA-type assays as shown in Figure 8. Antigen coated plastic plates were incubated with various amounts of $V_{NP}\gamma\delta$ SNase protein and bound antibody was then detected by virtue of its nuclease activity. This was achieved by addition to the plate of a solution containing DNA and ethidium bromide. Following digestion of the DNA substrate by the immobilized $V_{NP}\gamma\delta$ SNase antibody, the fluorescence due to the DNA/ethidium bromide complex substantially decreased.

In particular, polyvinyl microtitre plates were coated with $(NIP)_{20}$ - bovine serum albumin (40µg ml). After blocking unreacted sites with BSA, dilutions of $V_{NP}\gamma\delta$ SNase or $V_{NP}\gamma\delta$ chimeric antibodies were incubated in the wells, the amounts of antibody being given in nanograms in Figure 8. After washing off unbound material, a solution (40µl) containing 1µg M13 single

stranded DNA and 1ug/ml ethidium bromide in pH 8·5 buffer was added. The plate was photographed after a 1 hour incubation at 37°C.

As shown in Figure 8, quantities in the range of 10 ng of $V_{NP}\gamma$ SNase antibody are easily detected and no decrease in fluorescence is obtained with the $V_{NP}\gamma\delta$ antibody control. We have found that the assay may be made at least tenfold more sensitive by increasing the incubation time with the DNA substrate.

Example 3 - Fab-myc chimeric antibody

The carboxyterminal portion of the mouse c-myc gene was fused to the antibody Fab. The product of the c-myc gene is a protein which contains many thiol groups and is normally resident within the cell. There is no reason to believe that the third exon of c-myc on its own will encode a functional protein domain. Thus, if the Fab-myc fusion protein were secreted from the cell it would provide a source of protein for making anti-myc antisera.

Plasmid pSV-$V_{NP}\gamma$ myc was assembled essentially as described for the assembly of pSV-$V_{NP}\gamma\delta$ except that the pSV-$V_{NP}\gamma\delta$ Sac I - Xho I fragment containing the $C\delta_s$ exon was replaced by a Sac I - Bgl II fragment containing the 3' exon of mouse c-myc. The restriction site in Figure 9 marked Bg/B is a site formed by joining the Bgl II site at the 3' end of c-myc to the Bam HI site of pSV2gpt. The c-myc fragment comes from phage λ MYG2, which contains the translocated c-myc gene of mouse plasmacytoma X63Ag8 (see Neuberger and Calabi, loc. cit).

The plasmid is similar in structure to pSV-$V_{NP}\gamma\delta$ except that the $C\delta_s$ exon is replaced with the 3'-terminal exon of the mouse c-myc gene. This c-myc exon encodes 187 aminoacids (see Bernard, O., Cory, S., Gerondatis, S., Webb, E. and Adams, J. M., EMBO J., 2375-2383, 1983) and should provide the transcription polyadenylation signal.

The plasmid was transfected into J558L and cells from wells positive for production of anti-NP antibody cloned by limiting dilution. Hapten-binding protein was purified from culture supernatants and analyzed for the presence of c-myc antigenic determinants in an indirect radioimmunoassay.

Samples of either the putative Fab-myc or of the anti-NP $F(ab)'_2$ (to act as control) were incubated in wells of a polyvinyl microtitre plate that had been coated with a monoclonal anti-c-myc antibody; bound anti-NP antibody was then detected using a radioiodinated monoclonal anti-idiotope antibody which recognises the Fab portion of the anti-NP antibodies.

As shown in Figure 10, the Fab-myc clearly binds to the monoclonal anti-c-myc antibody, whereas the anti-NP $F(ab)'_2$ and other controls do not. The Fab-myc was also recognized by two other monoclonal antibodies that are specific for the carboxyterminal end of c-myc. SDS/polyacrylamide gel electrophoresis of the Fab-myc reveals that it is somewhat heterogenous; a band comigrating with λ_1 light chains and several bands with higher molecular weight in the range 38,000 to 55,000 daltons are observed, without a single dominant heavy chain band being apparent. The expected size of the Fab-myc is 50,000 daltons. It has been observed that, after prolonged storage, precipitates appear in the Fab-myc sample and SDS/polyacrylamide gel electrophoresis reveals more extensive heterogeneity of the heavy chain bands. We therefore believe that the heterogeneity of the Fab-myc protein indicated by the SDS/polyarcylamide gel analysis is most probably due to proteolytic degradation.

Example 4 - Chimeric Mouse - Human Antibodies.

A plasmid pSV-$V_{NP}H\gamma$ was constructed as follows. The Eco RI - Bam HI fragment of plasmid pSV-Vμ1 was

cloned between the Eco RI and Bam HI sites of plasmid pSV2gpt to yield pSV-V_{NP}. The Bam HI fragment of phage λε1.2 (see Flanagan, J. G. and Rabbitts, T. H., EMBO J., 1, 655-660, 1982), which includes exons Cε1 to Cε4 of the human ε gene was cloned into pSV-V_{NP} and the plasmid pSV-V_{NP}Hε completed by including in its unique Eco RI site the Xba I - Eco RI enhancer-containing fragment of the mouse heavy chain locus.

The structure of the pSV-V_{NP}Hε plasmid is shown in Figure 11 and encodes for a heavy chain comprising a mouse variable region and a human ε constant region.

pSV-V_{NP}Hε was introduced into J558 cells by spheroplast fusion, and stably transfected clones were selected as described above. Transfectants were obtained with a frequency of between 10^{-3} and 10^{-4}. Culture supernatants were assayed for production of λ1 - bearing anti-NP antibodies by radioimmunoassay as described above. Between 50 and 80% of the clones were positive. Antibody was purified from culture supernatants of several transfected clones by affinity chromatography as described above, with yield of about 2mg per litre.

Binding inhibition assays were used to demonstrate that the purified chimeric antibody displayed human antigenic determinants (Figure 12).

Assays were performed in which the binding of a radioiodinated human myeloma IgE to either a monoclonal (a) or polyclonal (b) anti-human ε antiserum was inhibited by various concentrations of unlabelled chimeric IgE (●) of unlabelled myeloma IgE (■).

Wells of a Dynatec microtitre plate were coated with a solution containing either 1 $\mu g.ml^{-1}$ of a monoclonal mouse anti-human ε antibody (antibody RB6-2; given by M. D. Cooper) or 3 $\mu g\ ml^{-1}$ of a polyclonal sheep anti-human ε antiserum (Seward Laboratory). After blocking of unreacted sites with bovine serum albumin, a human

myeloma IgE (Serotec) that had been radiolabelled with ^{125}I was incubated in the wells in the presence of different concentrations of either chimeric IgE or unlabelled myeloma IgE itself.

The affinity-purified chimeric antibody competes with the binding of radiolabelled human myelona IgE to both monoclonal (Figure 12A) and polyspecific (Figure 12B) anti-εantisera. Furthermore, binding of radiolabelled myeloma IgE to the anti-ε antiserum was inhibited completely (Figure 12B), indicating that the chimeric antibody displays all the ε antigenic determinants recognised by this polyclonal antiserum. The chimeric antibody competed with the binding of the radiolabelled myeloma IgE to the anti-ε antisera better than did the unlabelled myeloma IgE itself, probably because the purified chimeric antibody is essentially homogenous (see Figure 13), whereas the commercial sample of myeloma IgE is not.

The structure of the chimeric protein was investigated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Examination of reduced anti-NP IgE reveals a band comigrating with mouse λ1 light chains and a diffuse heavy-chain band of relative molecular mass (M_r) $\sim$ 72,000 (72K) (Figure 13A); this is considerably larger than the M_r of the ε polypeptide chain as predicted from the DNA sequence. However, this discrepancy is resolved by SDS-PAGE of biosynthetically labelled anti-NP IgE secreted by cells labelled in the presence of tunicamycin (Figure 13B). Incubation with this glycosylation inhibitor reduces the apparent relative molecular mass of the secreted anti-NP IgE from 72K to the predicted value of 62K. Thus, the chimeric IgE, like IgE from human myeloma, is heavily glycosylated. The electrophoretic mobility of unreduced anti-NP IgE in SDS polyacrylamide gels is consistent with

it having an $\varepsilon_2\lambda_2$ structure.

Figure 13A shows the SDS-PAGE results obtained from purified antibody (30µg) from a cloned pSV-$V_{NP}H\varepsilon$ transfectant of J558L (JW8/5/13) which was boiled in the presence of 2-mecaptoethanol, subjected to electrophoresis through a 9% gel and stained with Coomassie blue. In Figure 13B, the results are shown for biosynthetically labelled antibody from either J558L or the transfectant JW8/5/13 which was purified on NIP-Sepharose columns and analysed on a 7% gel after reduction. Samples purified from cells labelled in the presence of tunicamycin are marked +Tm. The positions of relative molecular mass markers are indicated (94K = relative molecular mass 94,000). Biosynthetic labelling with [L-^{35}S] methionine and purification of both labelled and unlabelled samples on hapten-Sepharose columns were performed as described above.

Two types of assay were performed to determine whether the chimeric anti-NP IgE antibody exhibits the physiological effector functions of authentic human IgE. In one type of assay, the ability of the antibody to trigger histamine release from human basophils was tested. A preparation of mononuclear cells from peripheral blood containing 1-2% basophils was passively sensitized with the chimeric antibody before incubation with hapten (5-iodo-4-hydroxy-3-nitrophenacetyl caproate, NIP) coupled to bovine serum albumin (NIP-BSA). The results (Table 1) indicate that, after preincubation of basophils with the chimeric IgE, NIP-BSA is able to trigger a dose-dependent release of histamine. A heterologous antigen, antigen P1 of the house dust mite Dermatophagoides pteronyssinus, failed to induce histamine release. Similarly, only background levels of histamine release were obtained with either the chimeric antibody or NIP-BSA alone. These results indicate that

the anti-NP IgE, like authentic human IgE, will not cause degranulation by itself, but will trigger histamine release following crosslinking with antigen.

In a second assay, it was demonstrated that incubation with anti-NP IgE could also block the subsequent passive sensitization of basophils by atopic sera containing high IgE levels. Table 2 shows that house dust mite antigen P1 can induce the release of histamine from basophils preincubated with serum containing anti-PI IgE antibody. However, this same serum failed to effect histamine release when the cells had been incubated previously with the chimeric antibody at concentrations >0.1 $\mu g\ ml^{-1}$.

The results described here demonstrate that transfection of DNA into mouse myeloma cells is an effective way of producing large amounts of chimeric antibodies in which mouse V regions provide antigen-binding specificity and human C_H regions provide human effector functions. The known antigen-binding specificty of such an antibody makes its purification extremely simple. Production of a chimeric IgE in this way has proved particularly attractive as no monoclonal human IgE of known antigen specificity was previously available. The chimeric antibody may, therefore, prove useful in routine clinical assays. It has been demonstrated that this monoclonal IgE is able to block the release of histamine from human basophils which can be triggered in vitro by sera from allergic subjects. It will clearly be important to discover whether analogous blocking can be achieved in vivo using skin sensitization assays.

Table 1 Histamine release triggered from human basophils passively sensitized with chimeric IgE

Antigen	% Histamine release (±s.e.m.)
-	6.4 + 0.8
10μg ml^{-1} NIP-BSA	18.4 + 2.4
1μg ml^{-1} NIP-BSA	36.0 + 5.6
0.1μg ml^{-1} NIP-BSA	31.2 + 12.0
0.01μg ml^{-1} NIP-BSA	26.4 + 2.4
0.001μg ml^{-1} NIP-BSA	7.2 + 1.6
0.0001μg ml^{-1} NIP-BSA	5.2 + 1.2
20μg ml^{-1} Antigen P1	4.5 + 0*

Mononuclear cells were prepared from peripheral blood by sedimentation through dextran-EDTA; these preparations contained 1-2% basophils. Cells were incubated in duplicate at 37°C for 2 h in complete Tyrode's buffer with 1.5μg ml^{-1} chimeric anti-NP IgE. Cells were then centrifuged and resuspended in complete Tyrode's buffer containing the indicated concentrations of $(NIP)_{30}$-BSA. After 15 min, the histamine released into the supernatant was extracted and assayed fluorimetrically. The maximum histamine release that could be obtained by incubating the cells at 100°C was 62.5 ng ml^{-1}. The results are expressed as percentage histamine release; (antigen-induced histamine release [ng ml^{-1}]) x 100/maximum histamine release obtainable (ng ml^{-1}). The mean background percentage release obtained in repeated experiments was 6.0 ± 2.5. Both NIP-BSA (1.0μg ml^{-1}) and antigen P1 (20μg ml^{-1}) alone repeatedly failed to induce histamine release above background levels.

*Data obtained in a separate experiment using cells from the same donor.

Table 2 Blocking of histamine release by preincubation with chimeric IgE

Concentration ($\mu g\ ml^{-1}$) of chimeric anti-NP IgE in preincubation	Serum from allergic subject	Antigen P1	% Histamine release ($\pm$s.e.m.)
-	+	+	20.5 + 2.7
0.01	+	+	16.9 + 2.4
0.1	+	+	7.2 + 0
1	+	+	8.2 + 1.0
10	+	+	7.2 + 1.0
10	-	-	8.4 + 1.2
-	-	+	7.5 + 1.7

Mononuclear cells were prepared as described in Table 1. A 2-h incubation with different concentrations of chimeric anti-NP IgE was followed by a 2-h incubation with allergic serum (diluted 1:30 in complete Tyrode's), prior to a 15-min exposure to antigen P1 ($20\mu g\ ml^{-1}$). The allergic serum used had a total IgE level of 4,800IU ml^{-1}, and 1,600 BA units ml^{-1} of IgE specific for D. pteronyssinus antigen P1. The maximum histamine release obtained by incubating the cells used in this experiment at 100°C was 10.4 ng ml^{-1}.

Example 4 - Fab- γ - Klenow Chimeric Antibody.

A plasmid, pSV-V_{NP}γ Klenow, which encodes the heavy chain of a recombinant antibody in which the Klenow fragment of DNA polymerase I is fused to the Fab portion of a mouse IgG2b molecule, was assembled.

The construction of pSV-V_{NP}γ Klenow is analogous to that previously described for pSV-V_{NP}γ SNase. The coding region for the Klenow fragment of DNA polymerase I was obtained as a BamHI fragment by combining two plasmids, pCJ14 (C.M. Joyce and N.G.D. Grindley, PNAS USA. 80, 1830-1834, 1983) and pCJ89 (C.M. Joyce and N.G.D. Grindley, J. Bacteriol., 158, 636-643, 1984), in which BamHI linkers have been inserted on either side of the Klenow coding region. This BamHI fragment was converted into a Sal I fragment by use of linkers and inserted into the unique Xho I site of pSV-V_{NP}γ 2b (CH2, CH3).

This antibody/enzyme fusion gene contains a variable region, V_{NP}, such that association of the pSV-V_{NP}γ Klenow heavy chain with the mouse immunoglobulin λ light chain from the J558L cell line will form a binding site for the hapten 4-hydroxy-3-nitrophenacetyl (NP). The V_{NP} gene is linked to exons encoding the CH1, hinge and aminoterminal part of the CH2 domain of a mouse immunoglobulin γ 2b heavy chain. The DNA encoding the rest of CH2 and all of CH3 has been replaced by a fragment of the E. coli DNA polymerase I gene that specifies the 5'-3' polymerase and 3'-5' exonuclease activities (the Klenow fragment). This Fab-Klenow fusion gene was cloned into the plasmid vector pSV2gpt which provides a polyadenylation signal for the Fab-Klenow transcription unit and also provides a marker, gpt, that confers resistance to the drug mycophenolic acid and thus allows selection of stably transfected mammalian cells.

Spheroplast fusion was used as a means to introduce

pSV-$V_{NP}\gamma$1 Klenow DNA into J558L cells. (The procedure used for protoplast fusion and selection of transfected clones is described in detail by M. S. Neuberger and G. T. Williams in Protein engineering : applications in science, medicine and industry (M. Inouye and R. Sarma, eds.), Academic Press,) Stable transfectants were selected in medium containing mycophenolic acid and the presence of NP-specific antibody in the culture medium of such transfectants was identified by radioimmunoassay. The transfectants were cloned by limiting dilution and one particular clone, JW64/7, that gave a high antibody titre was chosen for further study.

The protein secreted by JW64/7 was examined by SDS/polacrylamide gel electrophoresis of biosynthetically labelled samples that had been purified on hapten-Sepharose sorbent (Figure 15B). As expected from the predicted structure of the Fab-Klenow protein (Figure 14B), the gel reveals the presence of two polypeptide chains: a band corresponding to λ_1 light chain and a heavy chain of mol. wt. about 96,000 (Figure 15B). Within the Klenow portion of the Fab-Klenow heavy chain, four sequences of the form Asn-X-Thr/Ser are encountered; these might constitute sites for N-linked glycosylation. In order to discover whether the Fab-Klenow heavy chain is in fact glycosylated, biosynthetic labelling experiments were performed in the presence of the glycosylation inhibitor tunicamycin. The results (Figure 15B) reveal that tunicamycin does indeed result in the synthesis of a heavy chain of reduced molecular weight.

Fab-Klenow protein was purified by affinity chromatography on NP-Sepharose from culture supernatant of JW64/7 grown in DMEM/10% foetal calf serum, giving a homogeneous preparation (Figure 15A). The yield varied in the range of 1 to 15 mg of protein per litre of culture supernatant. The 5'-3' polymerase activity of

the purified protein was measured using the classical assay (The assay was performed as described by P. Setlow, Methods Enzymol. 29, 3-12, 1974.) in which the enzyme is incubated with "activated" DNA and the four dNTPs, one of which is radiolabelled; the incorporation of radioactivity into acid-precipitable material is followed. In this assay, using activated calf thymus DNA as substrate, the Fab-Klenow gave an activity of about 1.1×10^3 units/mg as compared to a value of 7×10^3 units/mg obtained under the same assay conditions using a commercial sample of homogeneous Klenow fragment which had been prepared by proteolytic fragmentation of DNA polymerase I purified from E. coli. A decreased specific activity (expressed as units per mg protein) of the Fab-Klenow compared to the normal enzyme is to be expected in view of their different molecular weights. However, this can only account for part of the difference in activity. Examination of unreduced Fab-Klenow in SDS/polacrylamide gels (not shown) suggests the presence of divalent $F(ab')_2$-Klenow as well as monovalent protein. It is possible that the $F(ab')_2$-Klenow might only show half-site reactivity; alternatively, it might be that glycosylation reduces the specific activity of the Fab-Klenow.

A major use of DNA polymerase I Klenow fragment is in the chain termination of DNA sequencing. As shown in Figure 16, Fab-Klenow can indeed be used successfully for this purpose. Figure 16 shows the use of Fab-Klenow in chain termination DNA sequencing. DNA from an M13 clone was sequenced using Fab-Klenow as described by Sanger et al. (F. Sanger, S. Nicklen and A. R. Coulson, PNAS USA, 74, 5463-5476, 1077) using 1 unit per clone of Fab-Klenow.

Thus it has been shown that recombinant antibody technology can usefully be applied to the tagging of specific enzymes such that they are secreted from the

cell and can be readily purified to homogeneity in a one step purification.

CONCLUSION

The above Examples demonstrate that by using the process of the present invention, it is possible to produce secreted, functional chimeric antibodies, which was not previously possible. The process enables the production of a number of chimeric antibodies which have not previously been known.

CLAIMS

1. A process for the production of a chimeric antibody, comprising:

a) preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence comprising a first part which encodes at least the variable region of the heavy or light chain of an Ig molecule and a second part which encodes at least part of a second protein;

b) if necessary, preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable region of a complementary light or heavy chain respectively of an Ig molecule;

c) transforming an immortalised mammalian cell line with the or both prepared vectors; and

d) culturing said transformed cell line to produce the chimeric antibody.

2. The method of claim 1, wherein the immortalised cell line is of myeloid origin.

3. The method of claim 2, wherein the immortalised cell line is a myeloma cell line or derivative thereof.

4. The method of any one of claims 1 to 3, wherein the immortalised cell line secretes a complementary light or heavy chain respectively, whereby step b) is omitted.

5. The method of any one of claims 1 to 3, wherein step b) is carried out by preparing a separate vector which is transformed into the immortalised cell line with the vector prepared in step a).

6. The method of any one of claims 1 to 3, wherein step b) is carried out by further manipulation of the vector prepared in step a).

7. The method of any one of claims 1 to 4, wherein the immortalised cell line secretes a complementary light or heavy claim respectively, and the transformation is accomplished by transforming a suitable bacterial cell with the vector prepared in step a) and then fusing the bacterial cell with the immortalised cell line.

8. The method of any one of claims 1 to 7, wherein the first part of the DNA sequence of the vector prepared in step a) is directly linked to the second part.

9. The method of any one of claims 1 to 7, wherein the first part of the DNA sequence of the vector prepared in step a) is linked to the second part by an intervening sequence which encodes a specific cleavage sequence.

10. The method of claim 9, wherein the cleavage sequence is specific for Factor Xa.

11. A chimeric antibody comprising an antigen binding region having a known specificity and of a first class linked to at least part of a constant region derived from at least one Ig molecule of a different species, class or subclass.

12. The chimeric antibody of claim 11, which is a $F(ab)'_2$-type antibody.

13. The chimeric antibody of claim 11, wherein the variable region is derived from a mouse antibody specific for NP and the constant region is a human CE region.

14. A chimeric antibody comprising an antigen binding region linked to at least a part of an enzyme.

15. A chimeric antibody comprising an antigen binding region linked to a protein having a known binding specificity.

16. A chimeric antibody comprising an antigen binding region linked to a protein expressed by a known gene.

17. A chimeric antibody comprising an antigen binding region linked to a protein toxin.

18. The chimeric antibody of any one of claims 11 to 17 wherein the antigen binding region is connected to the second protein by a specifically cleavable linker sequence.

19. Use of the chimeric antibody of claim 14 in an ELISA-type assay.

20. Use of the chimeric antibody of claim 15 in an immunoassay.

21. The chimeric antibody of claim 17 for use in therapy.

22. Use of the chimeric antibody of claim 18 in the purification by affinity chromatography of the second protein.

23. The plasmic pSV-V_{NP} γδ.

24. The plasmid pSV-V_{NP} γ SNase.

25. The plasmid pSV-V_{NP} γ myc.

26. The plasmid pSV-V_{NP} Hε.

27. The plasmid pSV-V_{NP} γ Klenow.

28. J558L cells transformed with any one of the plasmids of claims 23 to 27.

29. The chimeric antibody produced by culturing any one of transformed J558L cells of claim 28.

FIG. 1B

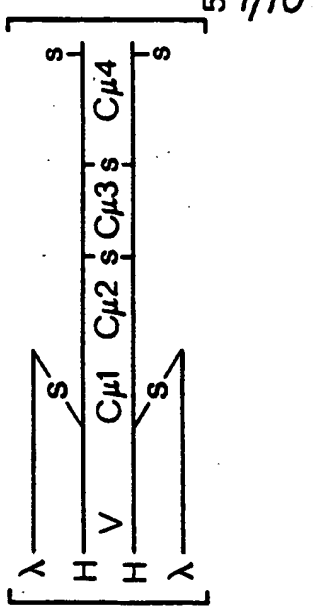


FIG. 3B

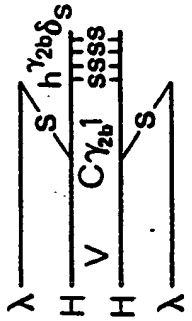


FIG. 1A

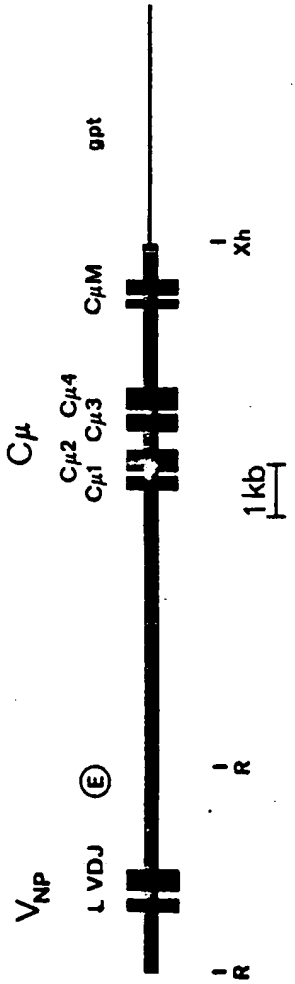
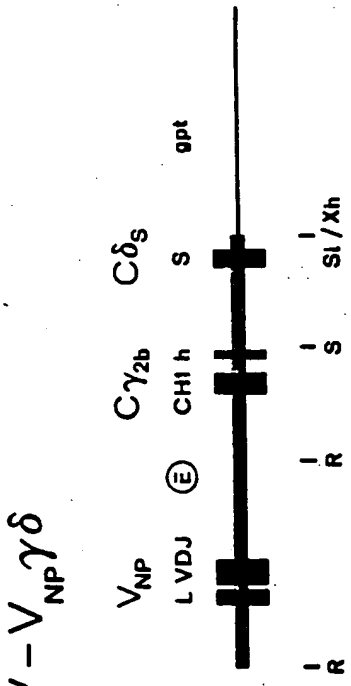


FIG. 3A



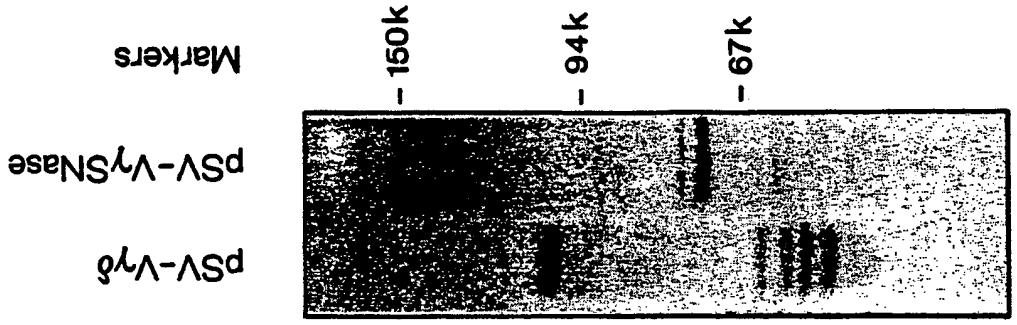


FIG. 4

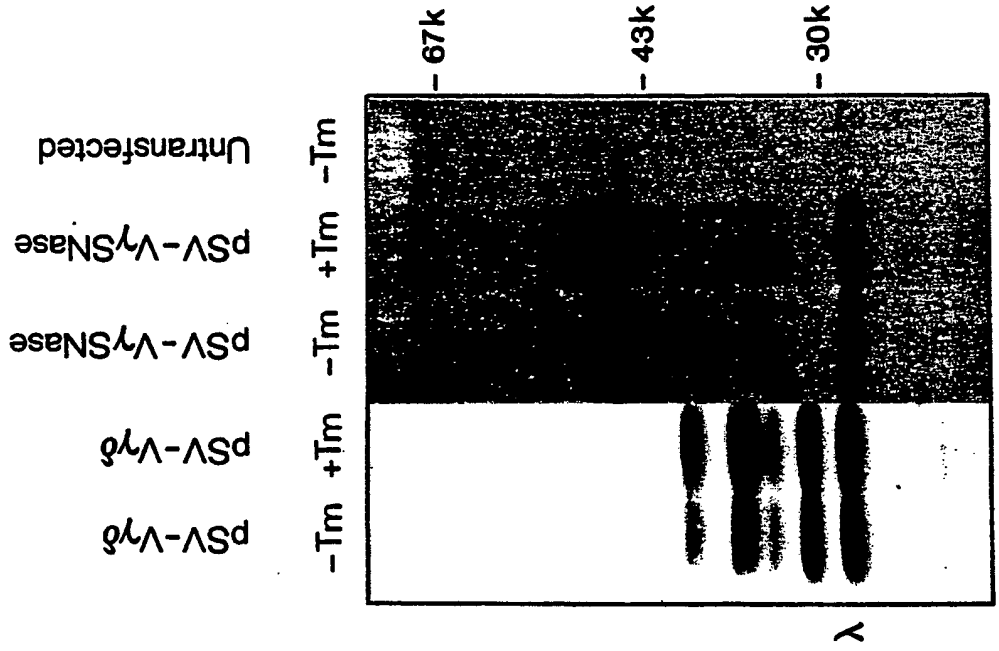


FIG. 5

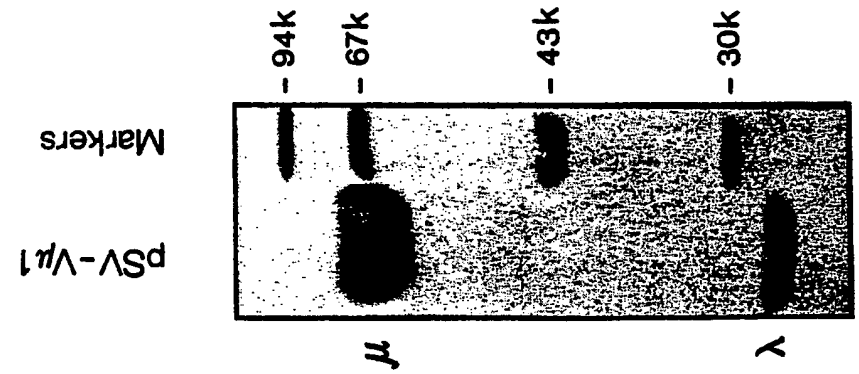


FIG. 2

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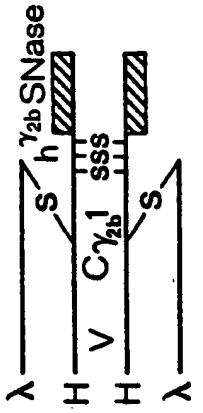


FIG. 6B

pSV-$V_{NP}\gamma$SNase

FIG. 6A

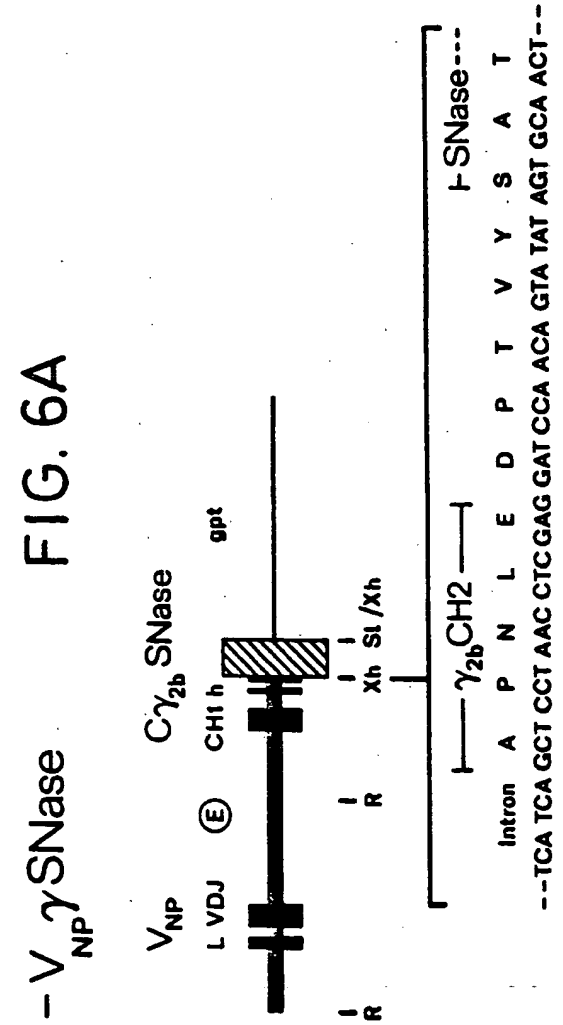
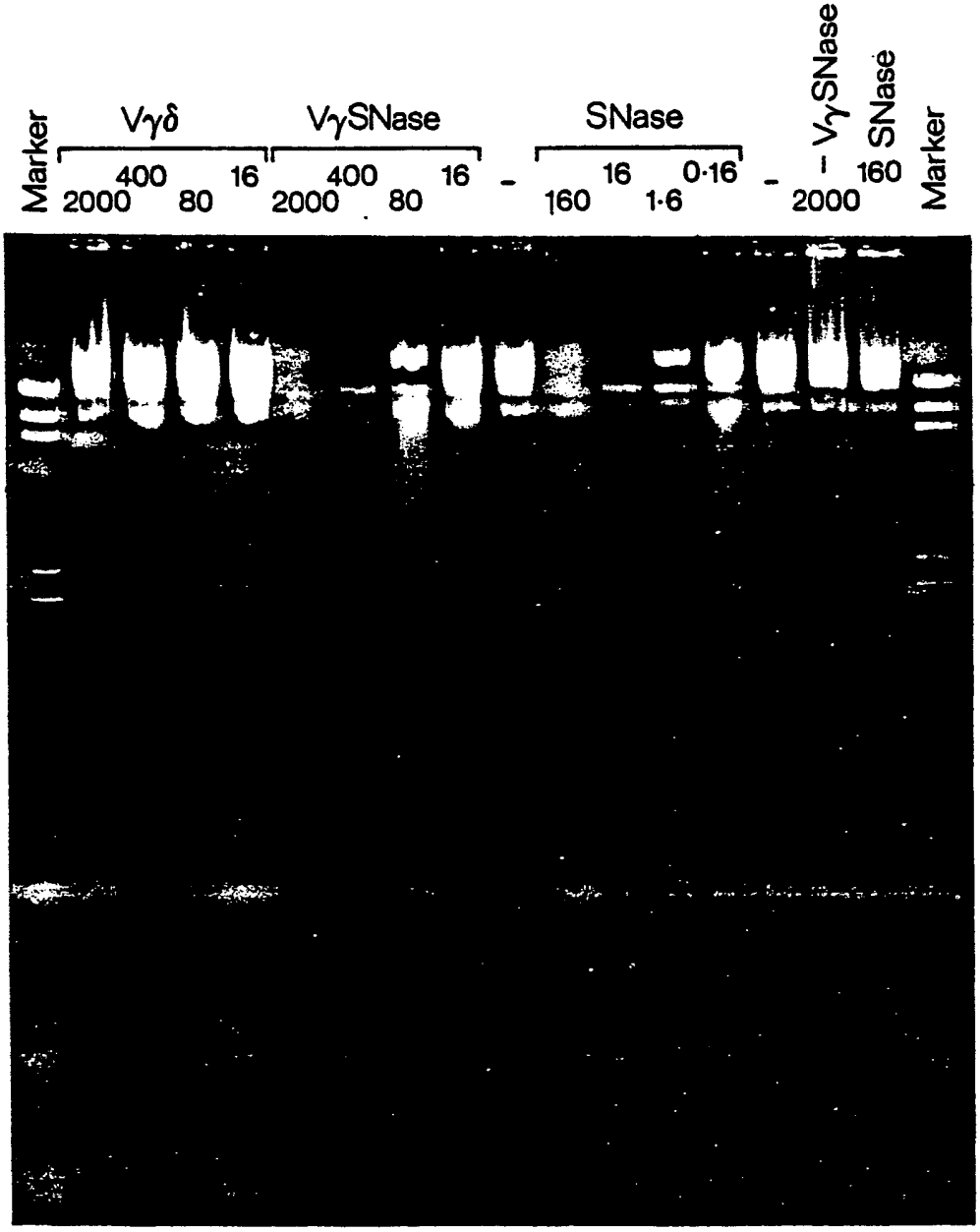


FIG. 7


Marker
Vγδ
VγSNase
SNase
VγSNase
SNase
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0·16
2000
160

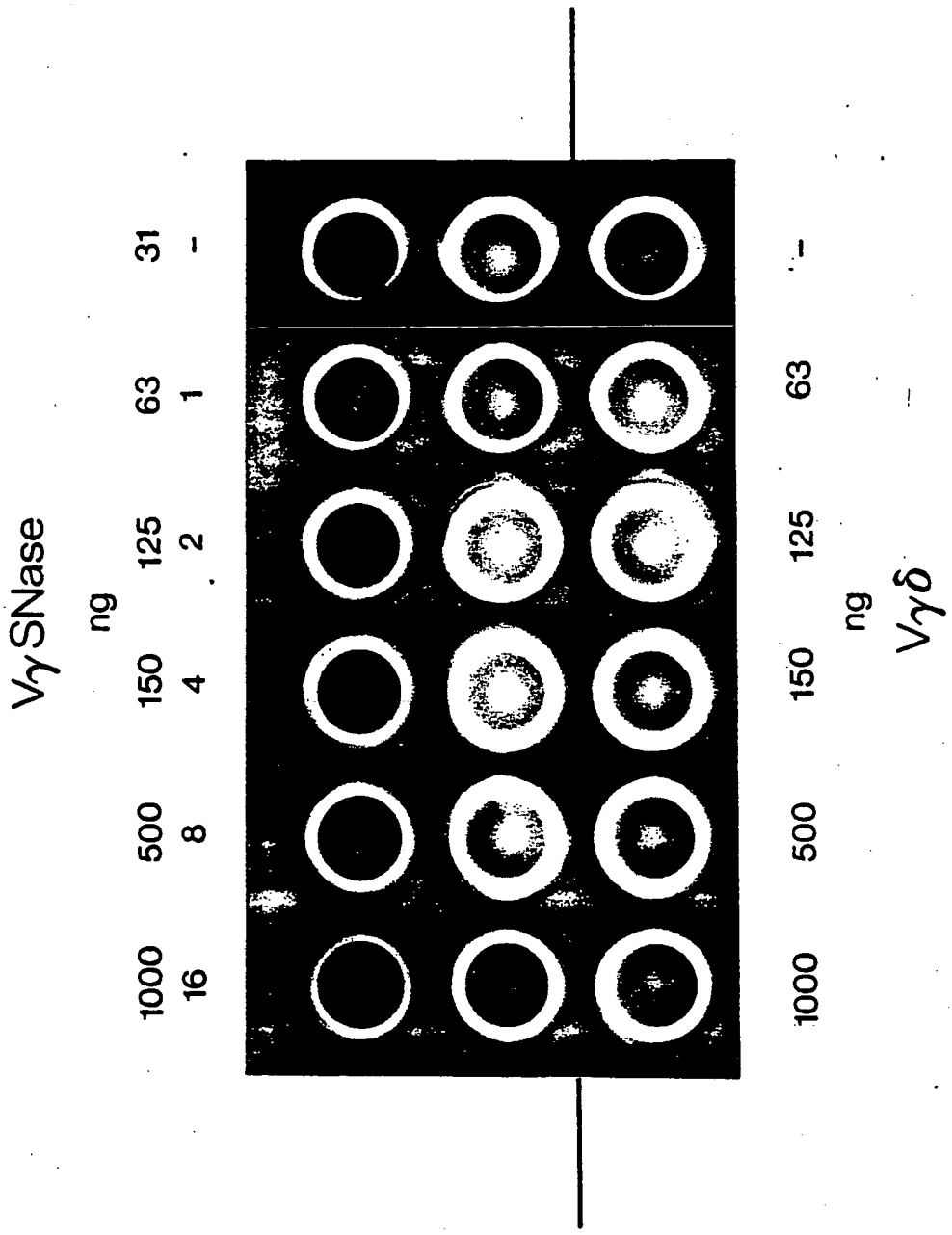
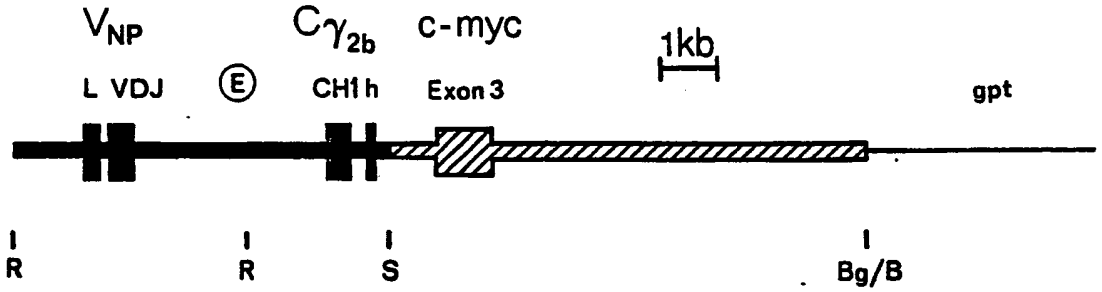

Vγ SNase
ng
31
63
125
150
500
1000
–
1
2
4
8
16
Vγδ
ng
–
63
125
150
500
1000

FIG. 8

FIG. 9

pSV – $V_{NP}\gamma$myc



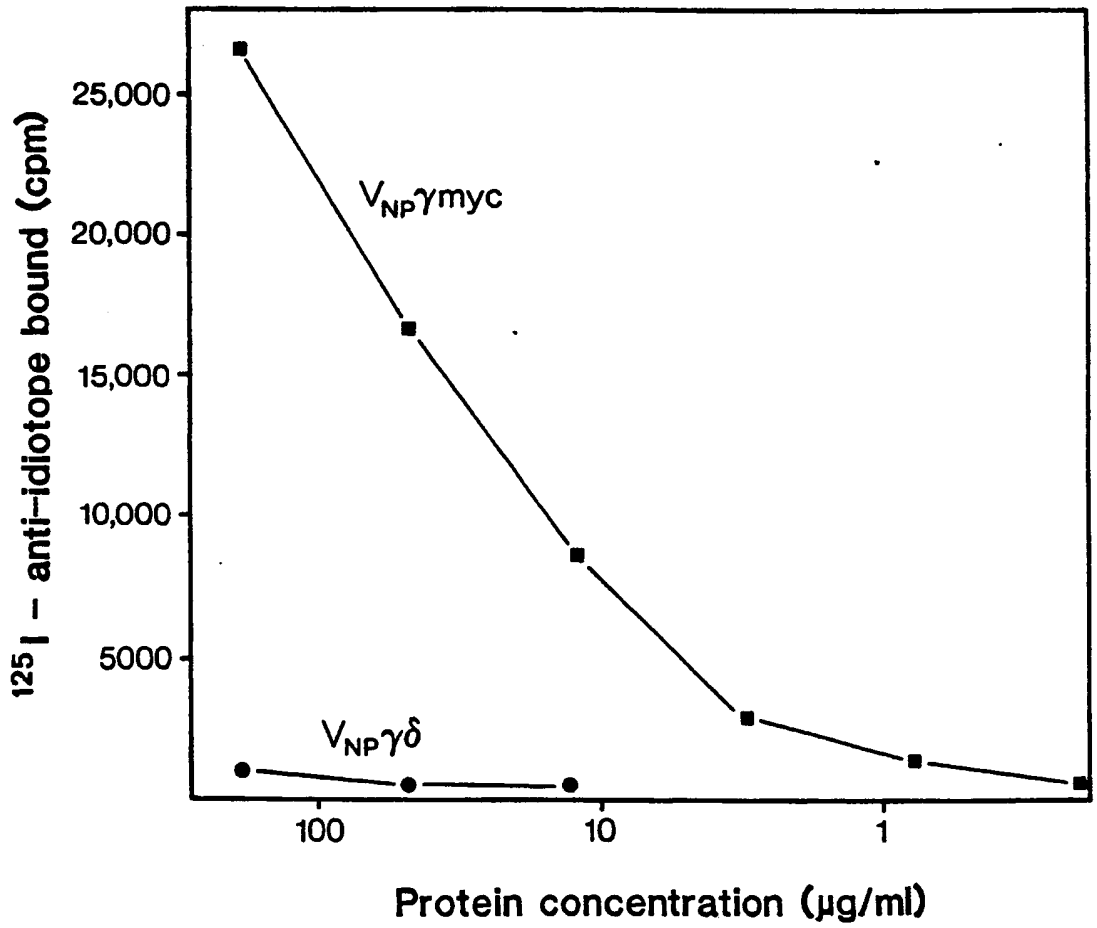
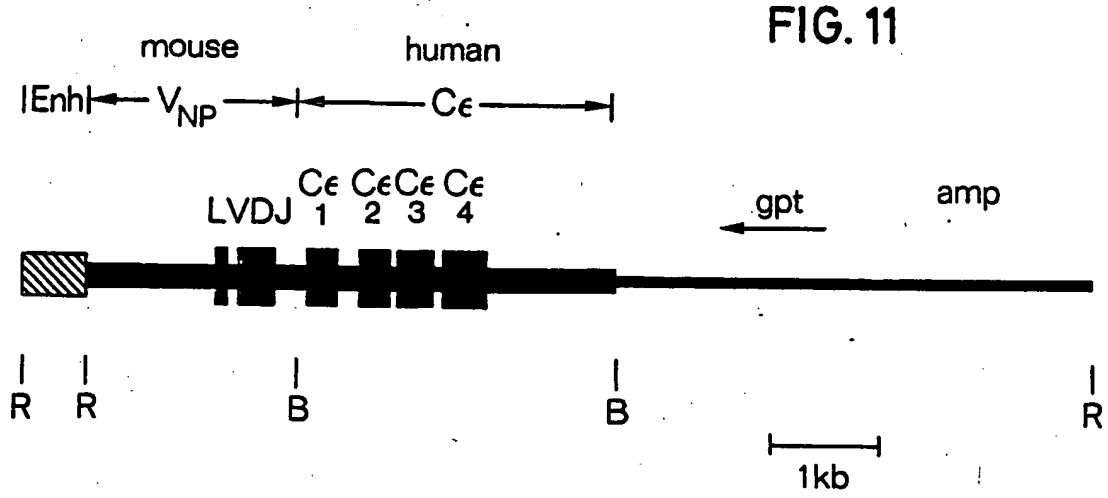


FIG. 10

pSV - V_{NP} Hε

FIG. 11



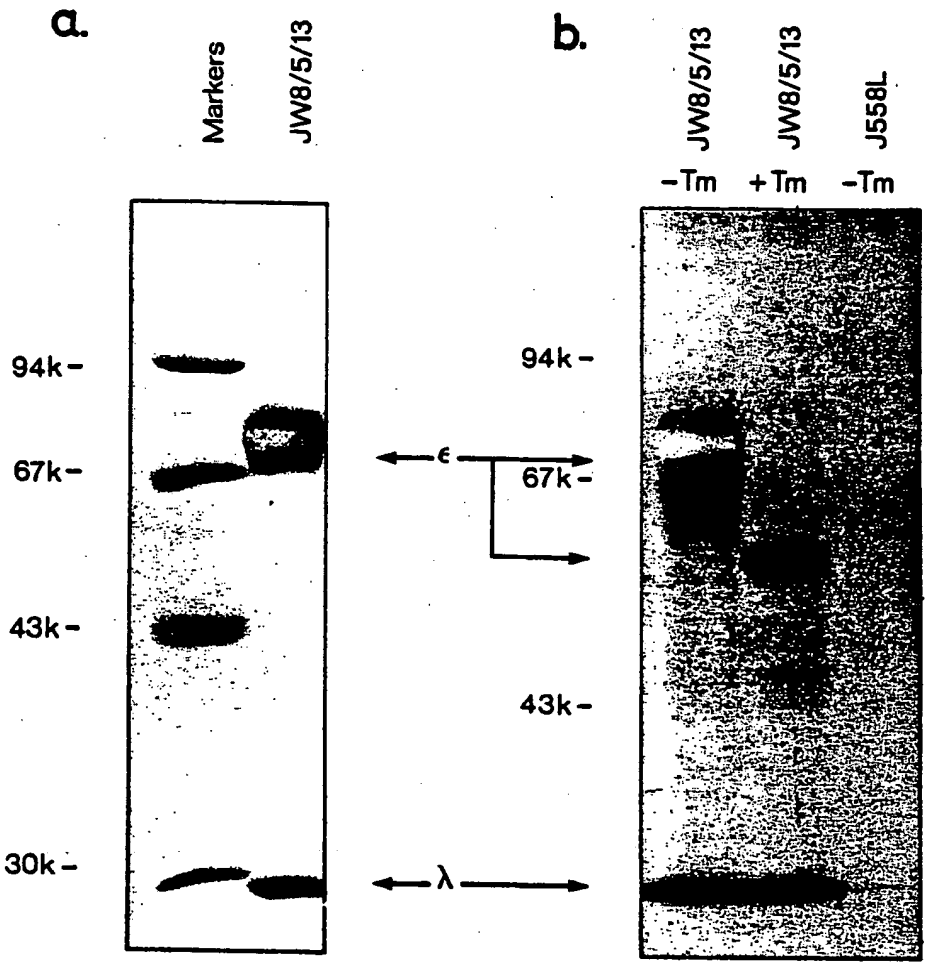


FIG. 13

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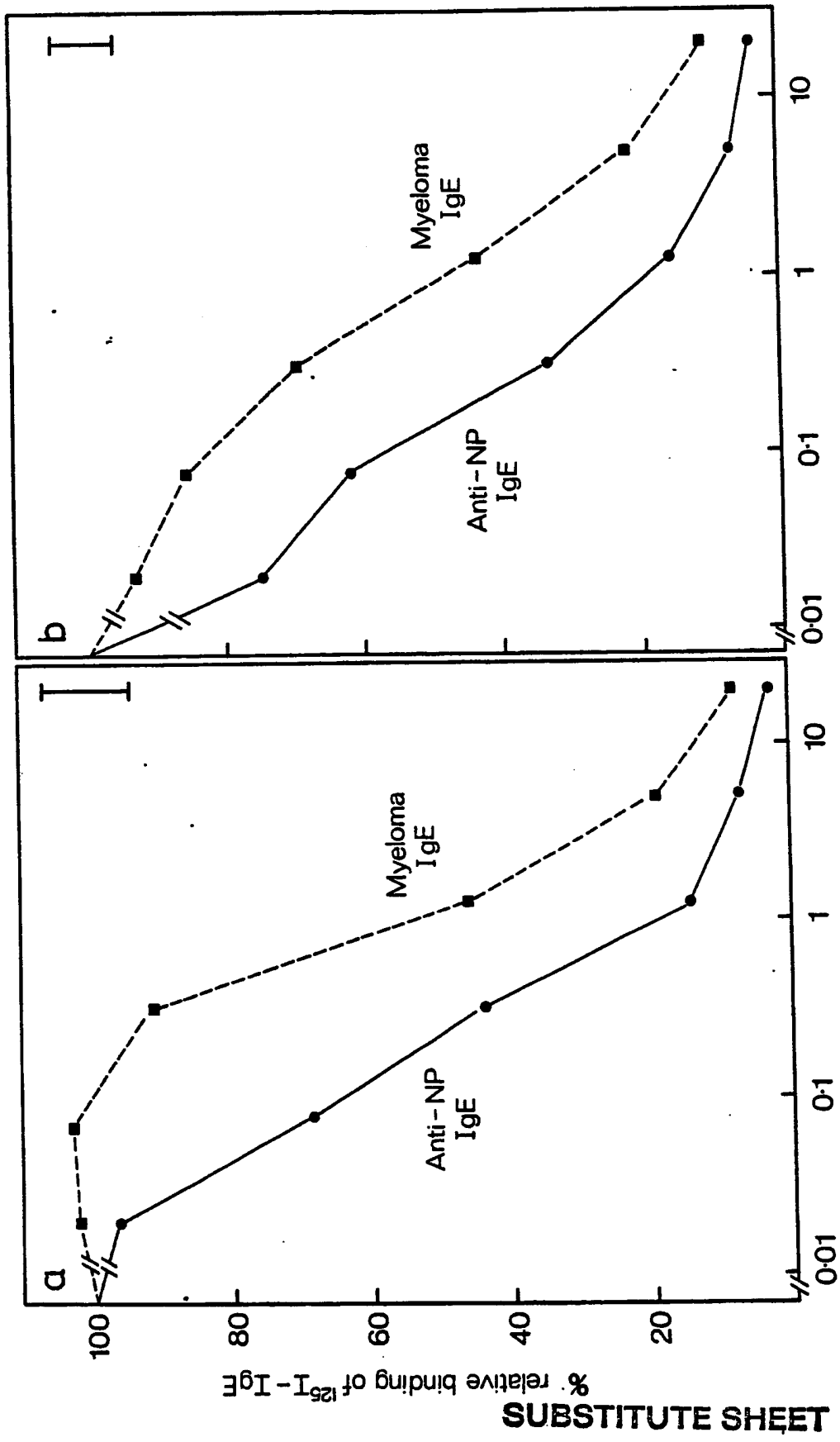
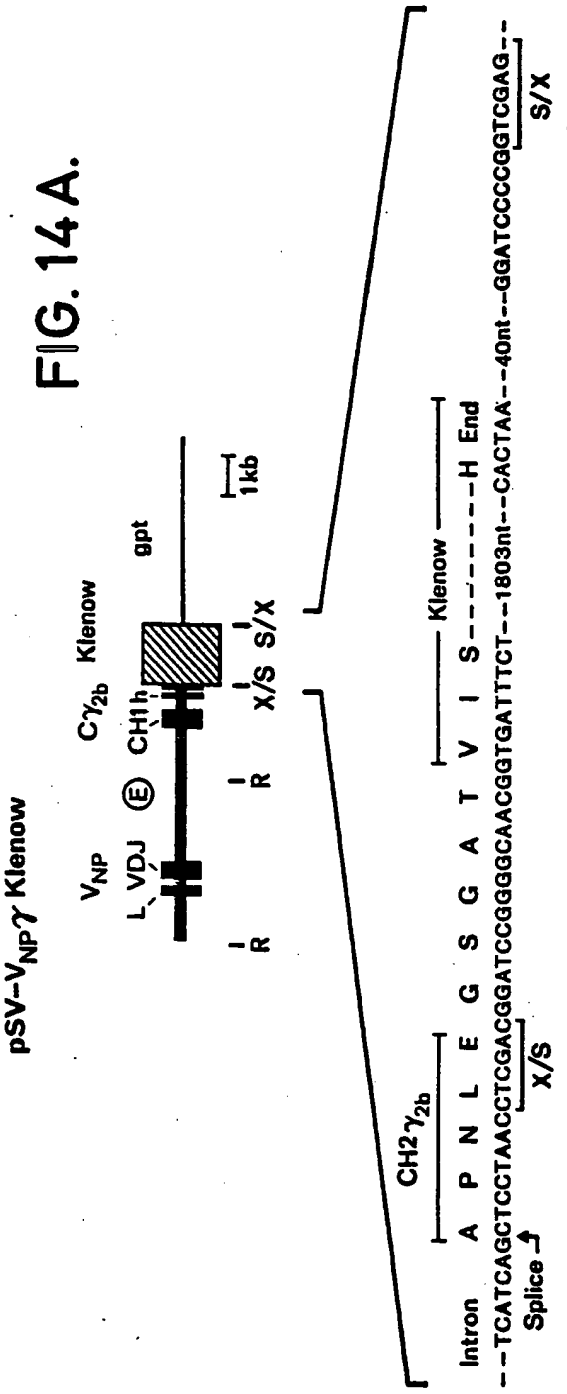

a
b
Myeloma IgE
Anti-NP IgE
% relative binding of ^{125}I-IgE
100
80
60
40
20
0·01
0·1
1
10
Inhibitor concentration (µg/ml)

FIG. 12

FIG. 14 A.



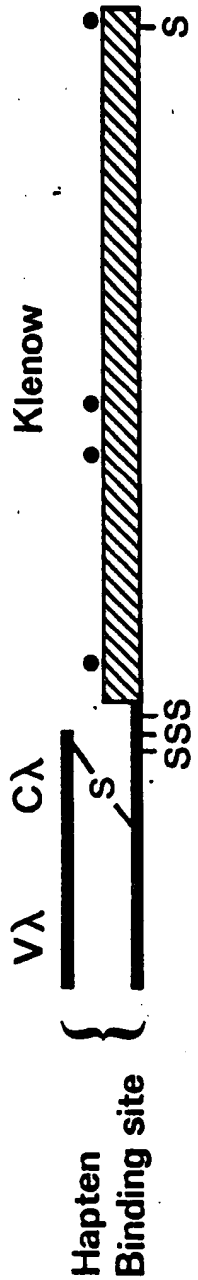


FIG. 14 B.

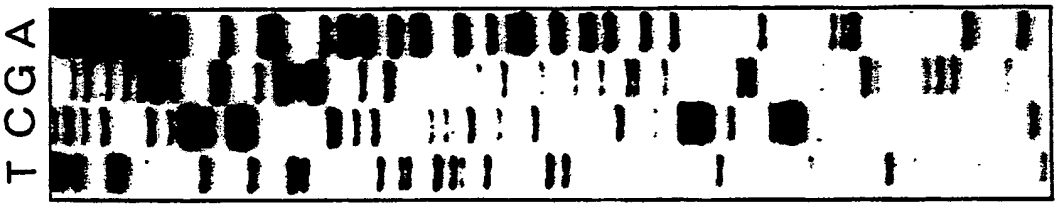

T C G A

FIG.16

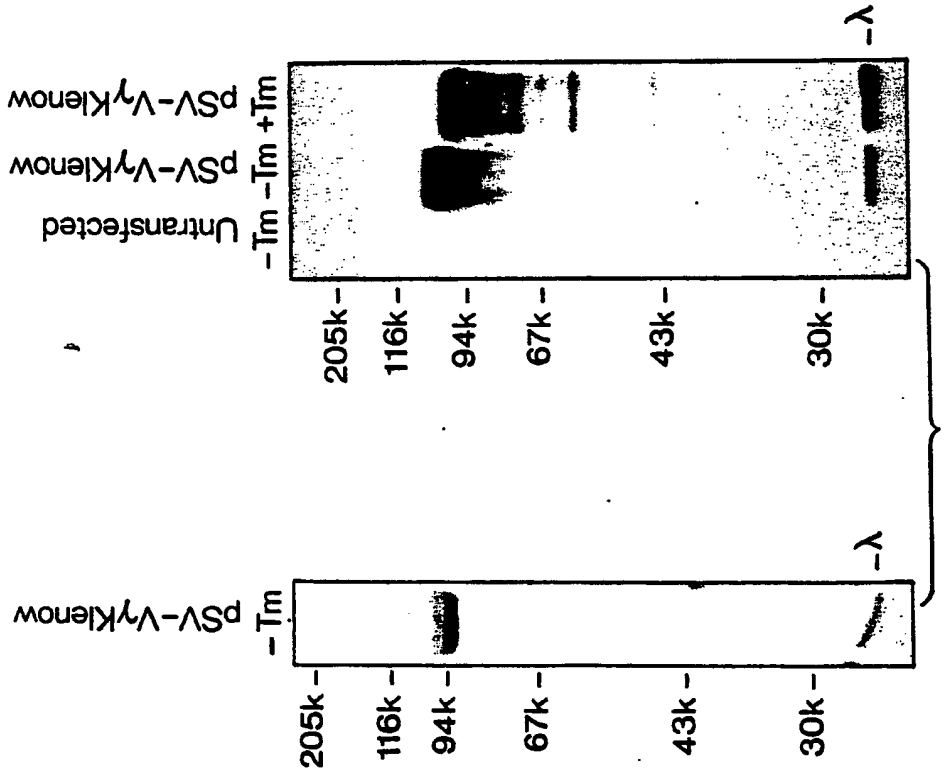

pSV-Vγ Klenow
-Tm
205k
116k
94k
67k
43k
30k
γ-
Untransfected
-Tm
-Tm +Tm
pSV-Vγ Klenow
pSV-Vγ Klenow
205k
116k
94k
67k
43k
30k
γ-

FIG. 15

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 85/00392

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; C 07 K 15/00; A 61 K 39/395; C 07 K 3/18; C 12 N 5/00; C 12 P 21/00; G 01 N 33/563

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0068763 (THE BOARD OF REGENTS UNIVERSITY TEXAS SYSTEM) 5 January 1983, see claims 1-15	15
A		1
A	Chemical Abstracts, volume 95, no.4, 27 July 1981, Columbus, Ohio (US) V. Raso et al.: "Hybrid antibodies with dual specificity for the delivery of ricin to immunoglobulin-bearing target cells", see page 363, abstract 30711m, & Cancer Res. 1981, 41(6) 2073-8 (Eng.)	1,17
Y	EP, A, 0105521 (TAKEDA) 18 April 1984, see claims 1-15	1
Y	WO, A, 83/03971 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 24 November 1983, see claims 1-13; pages 4-8	1
A	EP, A, 0090898 (GENETIC SYSTEMS CORP.) 12 October 1983, see claims 1-10	1

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6th December 1985	20 DEC. 1985
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	G.L.M. Kruydenberg

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,X	Nature, 314, no. 6010, April 1985 London (GB) Shun-Ichi Takeda et al.: "Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences", pages 452-454, see the whole article	1,11,12
P,X	Nature 314, no. 6008, 21st March 1985 London (GB) M. Neuberger et al.: "A hapten-specific chimaeric Ig E antibody with human physiological effector function", pages 268-270, see the whole article	1-12
P,X	Nature 312, no. 5995, December 1984 London (GB) G. Boulianne et al.: "Production of functional chimaeric mouse/human antibody", pages 643-646, see the whole article	1,11,12
P,X	Nature 312, no. 5995, December 1984 London (GB) M. Neuberger et al.: "Recombinant antibodies possessing novel effector functions", pages 604-608, see the whole article	1-8,14
P,X	EP, A, 0125023 (GENENTECH) 14 November 1984, see claims 1-21	1,11
P,X	EP, A, 0120694 (CELLTECH) 3 October 1984, see claims 1-33	1,11
A	WO, A, 84/00382 (NEVILLE) 2 February 1984, see claims 1-5	1
P,X	EP, A, 0150126 (IMMUNEX) 31 July 1985, see claims 1-19	1,14

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 85/00392 (SA 10580)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/12/85

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0068763	05/01/83	JP-A-	58059994	09/04/83
		US-A-	4474893	02/10/84
		CA-A-	1190873	23/07/85
EP-A- 0105521	18/04/84	JP-A-	59065099	13/04/84
WO-A- 8303971	24/11/83	AU-A-	1706283	02/12/83
		EP-A-	0108146	16/05/84
EP-A- 0090898	12/10/83	JP-A-	58201723	24/11/83
		US-A-	4464465	07/08/84
		CA-A-	1187010	14/05/85
EP-A- 0125023	14/11/84	AU-A-	2642984	11/10/84
		JP-A-	60155132	15/08/85
EP-A- 0120694	03/10/84	WO-A-	8403712	27/09/84
		GB-A-	2137631	10/10/84
		JP-T-	60500892	20/06/85
WO-A- 8400382	02/02/84	EP-A-	0100904	22/02/84
		US-A-	4500637	19/02/85
		US-A-	4520226	28/05/85
EP-A- 0150126	31/07/85	JP-A-	60222429	07/11/85

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Internationales Büro
INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 5 :		(11) Internationale Veröffentlichungsnummer: WO 93/13302
F01M 11/06, 1/12	A1	(43) Internationales Veröffentlichungsdatum: 8. Juli 1993 (08.07.93)

(21) Internationales Aktenzeichen: PCT/DE91/01001

(22) Internationales Anmeldedatum: 23. Dezember 1991 (23.12.91)

(71)(72) Anmelder und Erfinder: ZOCHE, Michael [DE/DE]; Keferstr. 13, D-8000 München 40 (DE).

(74) Anwalt: HAFT, BERNGRUBER, CZYBULKA; Hans-Sachs-Str. 5, Postfach 14 02 46, D-8000 München 5 (DE).

(81) Bestimmungsstaaten: JP, US, europäisches Patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

Veröffentlicht
Mit internationalem Recherchenbericht.

(54) Title: ENGINE WITH OIL SEPARATOR

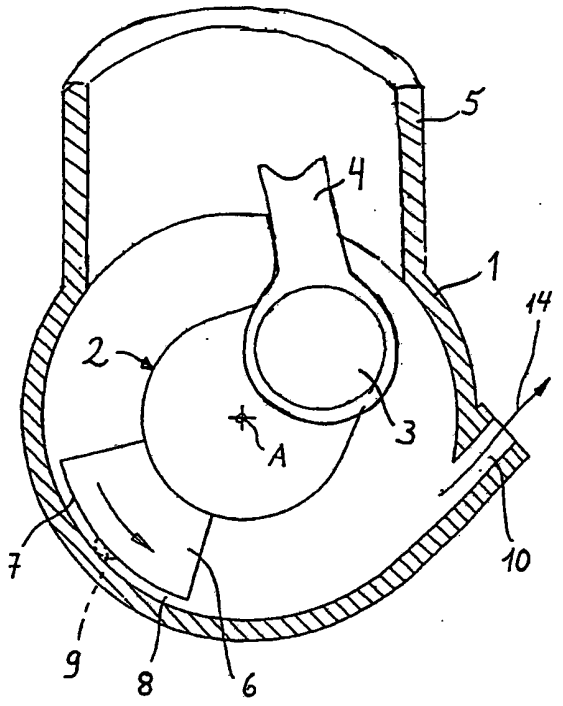
(54) Bezeichnung: MOTOR MIT EINER VORRICHTUNG ZUR ENTÖLUNG

(57) Abstract

In order to remove oil from the engine, i.e. to evacuate lubricant oil from the crankcase (1), the crankcase and the rotating parts (6, 2, 3) therein are designed so that a centrifugal flow is generated in the crankcase (1) and is used to separate the engine oil from the air that surrounds it. Preferably, only a small gap (8) remains between the outermost partially cylindrical rotating parts, in particular the counterweight (6), and the inner wall of the cylindrical crankcase (1). The oil is removed through an outlet (10) that extends tangentially out of the crankcase (1).

(57) Zusammenfassung

Zur Entölung des Motors, d.h. zum Abführen des Schmieröles aus dem Kurbelgehäuse (1) werden dieses und die darin rotierenden Teile (6, 2, 3) so ausgestaltet, daß sich in dem Kurbelgehäuse (1) eine Zentrifugalströmung einstellt, die zur Separierung des Motoröles und der dieses umgebenden Luft ausgenutzt wird. Vorzugsweise verbleibt zwischen den äußersten teilzylindrisch ausgebildeten rotierenden Teilen, insbesondere dem Gegengewicht (6) und der Innenwand des zylindrischen Kurbelgehäuses (1) ein nur geringer Spalt (8). Das Öl wird aus einer tangential aus dem Kurbelgehäuse (1) herausführenden Abflußöffnung (10) entfernt.



LEDIGLICH ZUR INFORMATION

Code, die zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AT	Österreich			MR	Mauritanien
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DK	Dänemark	MG	Madagaskar	US	Vereinigte Staaten von Amerika
ES	Spanien	ML	Mali	VN	Vietnam
FI	Finnland	MN	Mongolei		

Motor mit einer Vorrichtung zur Entölung

Ein Problem bei Motoren ist eine sichere Entölung des Motorengehäuses in jeder Betriebslage des Motors. Üblicherweise wird das Motoröl im sogenannten Sumpf, d.h. "unten" im Kurbelgehäuse, außerhalb der drehenden Teile des Motors, wie Kurbelwelle, Ausgleichsgewichte etc., gesammelt. Eine solche Entölung ist z.B. kritisch bei Flugmotoren für kunstflugtaugliche Flugzeuge, die sich längere Zeit etwa in Rückenlage aufhalten, aber auch bei herkömmlichen Automotoren, wenn durch Querbeschleunigungen Öl in den Bereich der drehenden Teile gelangt. Bei der Entölung sollte aus schmiertechnischen Gründen eine möglichst luftfreie Entölung, d.h. Förderung des Öles aus dem Kurbelgehäuse, gewährleistet sein, so daß kein Schaum im Öl gebildet wird.

Es wurde versucht, die Entölung durch zusätzliche Pumpen sicherzustellen. Zum einen ist dieses mit erheblichem Aufwand verbunden und gewährleistet zudem keine luftfreie Förderung des Öles, da die Pumpen, die stets laufen, bei nur geringer Ölmenge zwangsläufig auch Luft fördern.

Es ist Aufgabe der Erfindung, eine Entölung mit Hilfe einer einfachen Konstruktion sicherzustellen, die gewährleistet, daß das Öl praktisch luftfrei und vollständig aus dem Kurbelgehäuse entfernt wird.

Dies wird gemäß der Erfindung dadurch sichergestellt, daß die in dem Kurbelgehäuse rotierenden Teile des Motors, also Kurbelwelle, Kurbelzapfen, Pleuel und Gegengewichte,

von dem Motorgehäuse in einem solchen Abstand umgeben werden, daß zwischen der die rotierenden Teile umschreibenden Fläche und dem Kurbelgehäuse nur ein kleiner Spalt verbleibt, so daß durch die rotierenden Teile eine Zentrifugalströmung in dem Kurbelgehäuse erzeugt wird, die zur Förderung des Öles ausgenutzt wird. Das Öl wird aus einer tangential aus dem Kurbelgehäuse abgehenden Abflußöffnung aus dem Kurbelgehäuse gefördert. Die auf das Öl wirkende Zentrifugalkraft ist wesentlich größer als die auf das Öl wirkende Schwerkraft, so daß die Abflußöffnung an jeder beliebigen Stelle des zylinderförmigen Kurbelgehäuses angeordnet werden kann. Durch die Zentrifugalkräfte werden Luft und Öl zwangsläufig voneinander separiert. Durch Dimensionierung des Abströmwiderstandes durch die Abflußöffnung kann man erreichen, daß nur Öl aus der Abflußöffnung austritt, Luft hingegen nicht.

Es ist im übrigen nicht notwendig, daß das Kurbelgehäuse formmäßig exakt an die sämtliche rotierenden Teile umschreibende Außenfläche angepaßt, d.h. insbesondere formmäßig an die Krängung der Kurbelwelle; vielmehr ist es ausreichend, wenn die Innenwand des Kurbelgehäuses etwa durchgängig zylinderförmig und das Kurbelgehäuse zu beiden Seiten der Kurbelwelle möglichst eng an den dort rotierenden Teilen abgeschlossen ist. Vorzugsweise ist für jeden Zylinder eine separate Abflußöffnung vorgesehen.

Die Erfindung ist in einem Ausführungsbeispiel anhand der schematischen Zeichnung näher erläutert. In dieser stellen dar:

Fig. 1 einen Querschnitt durch einen Teil eines Motors;

Fig. 2 eine schematische Seitenansicht eines Vierzylinder-Reihenmotors.

In Fig. 1 ist ein zylindrisches Kurbelgehäuse 1 für eine Kurbelwelle 2 mit Kurbelzapfen 3 gezeigt, an denen Pleuel 4 angelenkt sind, die zu nicht dargestellten Kolben führen, die in einem Zylinder 5 hin- und hergleiten. Die Kurbelwelle 2 dreht um ihre Wellenachse A. Die Kurbelwelle 2 ist mit Gegengewichten 6 für die Pleuel und den Kolben verbunden, wobei zwischen der teilzylindrischen Unterfläche 7 des Gegengewichtes 6 und der Innenwand des Kurbelgehäuses 1 ein geringer Spalt 8 verbleibt. Am Boden des Gegengewichts kann noch ein Gleiter oder Wischer 9 vorgesehen sein, der direkt auf der Innenwand des Kurbelgehäuses 1 bzw. in einer Nut in der Innenwand gleitet. Am zylindrischen Kurbelgehäuse ist ferner eine etwa tangential aus dem Kurbelgehäuse herausführende Abflußbohrung 10 vorgesehen.

In Fig. 2 ist schematisch die Seitenansicht eines Vierzylinder-Reihenmotors mit dem zylindrischen Kurbelgehäuse 1 und den vier Zylindern 5 dargestellt. Mit der Kurbelwelle ist ferner ein Schwungrad 11 mit einer Kupplung sowie ein Ventilator 12 zur Kühlung verbunden. Für jeden Kurbelraum ist eine Abflußöffnung 10 vorgesehen, die jeweils über Leitungen mit einem Sammelbehälter 13 für das Schmieröl des Motors verbunden sind.

Wenn sich in dem Kurbelgehäuse 1 Motoröl befindet, so wird durch die drehenden Teile der Kurbelwelle in dem zylinderförmigen Bereich des Kurbelgehäuses 1 eine Zentrifugalströmung erzeugt, durch die etwaiges in dem Kurbelgehäuse vorliegendes Motoröl aus der Abflußöffnung 10 ausgetrieben

wird, wie durch den Pfeil 14 angedeutet und in dem Behälter 13 gesammelt. Aus diesem Behälter wird das Öl als Schmieröl wiederum in das Kurbelgehäuse zurückgeführt. Das Öl wird im wesentlichen durch die in dem Kurbelgehäuse 1 erzeugte Zentrifugalströmung ausgetrieben und nicht unbedingt durch den mechanischen Kontakt mit dem Gegengewicht 6 bzw. dem Wischer 9.

Die geschilderte Entölung erfolgt in allen Lagen des Kurbelgehäuses 1.

Es ist auch nicht notwendig, daß das gesamte Kurbelgehäuse zylinderförmig ist; hier können lediglich einige Bereiche zylinderförmig gestaltet werden, die für die Entölung wichtig sind, in die dann durch die Strömung das Öl eingebracht und über Abflußbohrungen ausgetrieben wird. Wichtig ist insbesondere die Gestaltung des Gegengewichtes 6, da dieses in der Regel den größten Radius aufweist. Durch die Unterfläche 7 des Gegengewichtes wird auch die erwähnte, die rotierenden Teile im Kurbelgehäuse umschreibende Außenfläche bestimmt, die quasi als Fortsetzung der teilzylindrischen Unterfläche des Gegengewichtes 6 zu denken ist. Allerdings tragen zu der erwähnten Zentrifugalströmung auch die anderen im Kurbelgehäuse rotierenden Teile, so insbesondere auch die Kurbelzapfen bei. Für eine gute Entölung wird man die Breite des Gegengewichtes in Richtung der Wellenachse A möglichst groß wählen.

Die geschilderte Entölung ist für alle Arten von Motoren geeignet, sei es Dieselmotoren, Ottomotoren, Reihenmotoren oder auch Sternmotoren. Insbesondere für letztere können die rotierenden Teile und das Kurbelgehäuse entsprechend der Erfindung sehr gut ausgestaltet werden.

Patentansprüche

1. Motor mit einem Kurbelgehäuse (1), einer Kurbelwelle (2), Zylindern (5), an Kurbelzapfen (3) der Kurbelwelle (2) gelagerten Pleueln (4), in den Zylindern (5) geführten mit den Pleueln verbundenen Kolben und einer Vorrichtung zur Entölung des Motors, dadurch gekennzeichnet, daß zwischen Kurbelgehäuse (1) und der die in dem Kurbelgehäuse (1) rotierenden Teile (6, 2, 3) umschreibenden Außenfläche (7) ein nur geringer Spalt (8) vorgesehen ist, und daß eine Abflußöffnung (10) zum Abfördern des Motoröles aus dem Kurbelgehäuse (1) vorgesehen ist.

2. Motor nach Anspruch 1, dadurch gekennzeichnet, daß Kurbelgehäuse (1) und die darin rotierenden Teile (6, 2, 3) so gestaltet sind, daß sich durch die rotierenden Teile (6, 2, 3) eine Zentrifugalströmung einstellt, die zur Förderung des Motoröles und zur Separierung dieses Ölstromes von der umgebenden Luft ausgenutzt wird.

3. Motor nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß die Abflußöffnung (10) etwa tangential aus dem Kurbelgehäuse führt.

4. Motor nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß das Kurbelgehäuse (1) zumindest in den für die Abführung des Motoröles vorgesehenen Bereichen zylinderförmig ausgebildet ist, und daß dort zwischen der Innenwand des Kurbelgehäuses (1) und der Außenfläche der äußersten rotierenden Teile, insbesondere des Gegengewichtes (6) ein geringer Spalt (8) vorgesehen ist.

5. Motor nach Anspruch 4, dadurch gekennzeichnet, daß die Außenfläche des Gegengewichtes (6) teilzylindrisch ist.

6. Motor nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß mit der Kurbelwelle (2) und insbesondere mit der Außenfläche (7) des Gegengewichtes (6) ein Gleiter (9) verbunden ist, der auf der Innenwand oder in einer Nut des Kurbelgehäuses (1) läuft.

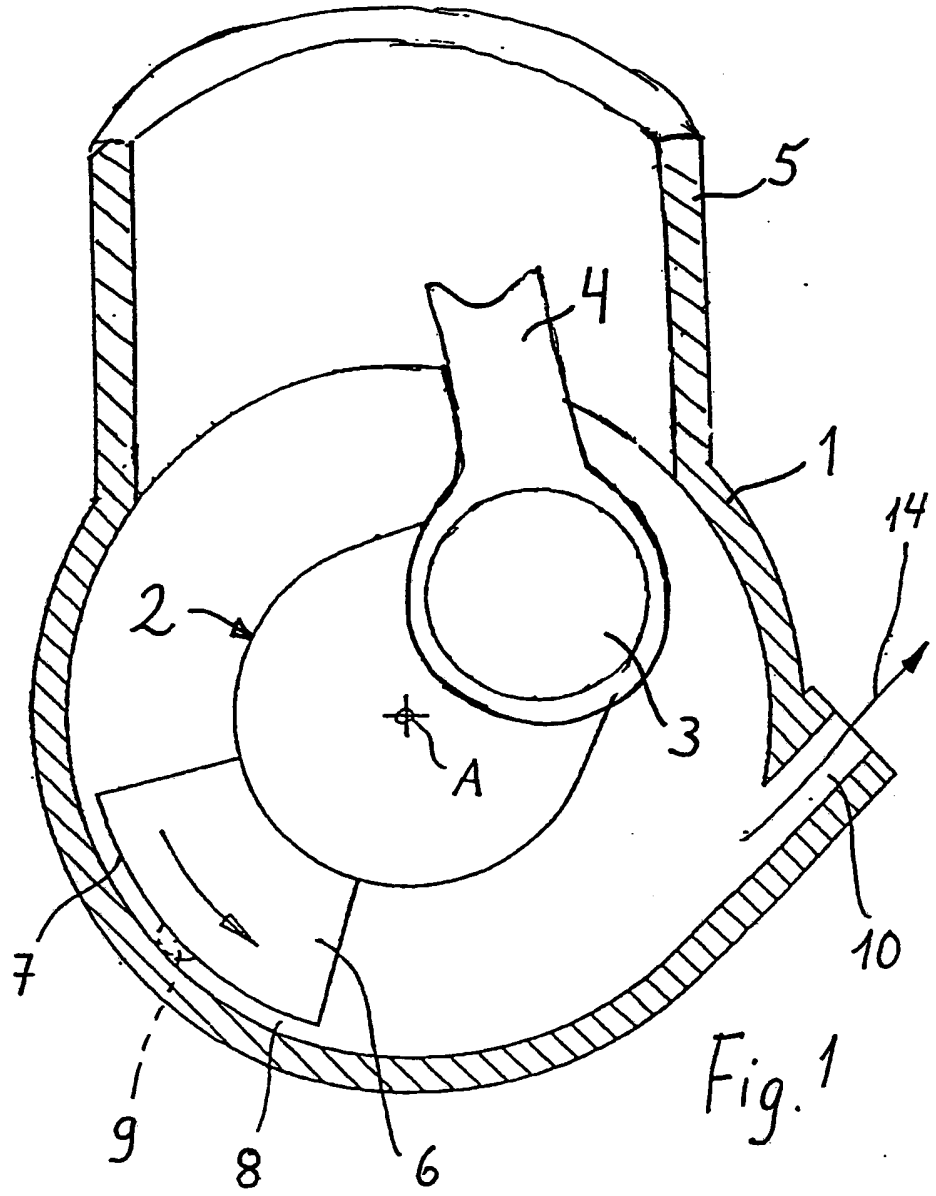

Fig. 1

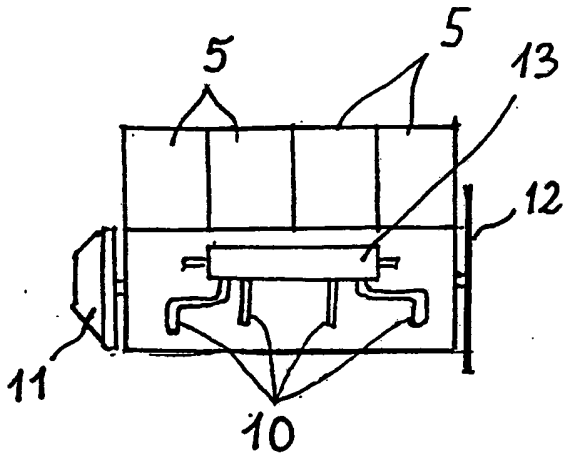

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DE 91/01001

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl.5: F 01 M 11/06; F 01 M 1/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl.5: F 01 M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FR, A, 618 367 (TISSERANT) 8 March 1927 see page 1, line 35 - page 2, line 83; figures	1,3-5
A	GB, A, 881 546 (SIMMS MOTOR UNITS LIMITED) 8 November 1961, see page 1, line 51 - page 2, line 33; figures	1,3,4,5
A	GB, A, 2539 a.d. 1912 (MCCURD) 14 November 1912 see figures	1
A	US, A, 3 045 411 (DALRYMPLE) 7 August 1953 see figures	2

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1992 (08.07.92)	31 July 1992 (31.07.92)
Name and mailing address of the ISA/ European Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. DE 9101001 SA 55069

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 08/07/92
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A-618367		None	
GB-A-881546		FR-A- 1256320 US-A- 3074625	
GB-A-		None	
US-A-3045411		None	

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen PCT/DE 91/01001

I. KLASSIFIKATION DES ANMELDUNGSGEGENSTANDS (bei mehreren Klassifikationssymbolen sind alle anzugeben)[6]

Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC

Int.Kl. 5 F01M11/06; F01M1/12

II. RECHERCHIERTE SACHGEBIETE

Recherchierter Mindestprüfstoff [7]

Klassifikationssytem	Klassifikationssymbole
Int.Kl. 5	F01M

Recherchierte nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Sachgebiete fallen [8]

III. EINSCHLAGIGE VEROFFENTLICHUNGEN [9]

Art.°	Kennzeichnung der Veröffentlichung[11], soweit erforderlich unter Angabe der maßgeblichen Teile[12]	Betr. Anspruch Nr.[13]
A	FR,A,618 367 (TISSERANT) 8. März 1927 siehe Seite 1, Zeile 35 - Seite 2, Zeile 83; Abbildungen	1,3-5
A	GB,A,881 546 (SIMMS MOTOR UNITS LIMITED) 8. November 1961 siehe Seite 1, Zeile 51 - Seite 2, Zeile 33; Abbildungen	1,3,4,5
A	GB,A, 2539 a.d. 1912 (MCCURD) 14. November 1912 siehe Abbildungen	1
A	US,A,3 045 411 (DALRYMPLE) 7. August 1953 siehe Abbildungen	2

° Besondere Kategorien von angegebenen Veröffentlichungen [10]:

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IV. BESCHEINIGUNG

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(54) Title: RECOMBINANT DNA-DERIVED CHOLERA TOXIN SUBUNIT ANALOGS

(57) Abstract

The development of subunits and subunit analogs of the cholera exotoxin by recombinant DNA techniques provides vaccine products that can retain their biological activity and immunogenicity, and can confer protection against disease challenge. Genetically-enginneered modifications of the subunits result in products that retain immunogenicity, yet are reduced in, or are essentially free of, enzymatic activity associated with toxin reactogenicity.

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RECOMBINANT DNA-DERIVED CHOLERA TOXIN SUBUNIT ANALOGS

BACKGROUND OF THE INVENTION

Field Of The Invention

The present invention relates to the recombinant expression of analog subunits of cholera exotoxin, and to vaccines based on such analogs. More particularly, genetically engineered modifications of the exotoxin provide analogs of cholera toxin having the capability to elicit a protective response with reduced or essentially no catalytic activity which can contribute to the reactogenicity of cholera vaccines.

Description Of The Art

The term "cholera" refers to the disease caused by infection with the etiologic agent *Vibrio cholerae*, most commonly occurring in geographical areas where poor hygienic conditions prevail. Cholera remains a major cause of morbidity and mortality in many parts of the world(1,2). Experience has shown that contraction of the disease usually confers long-lasting protection against subsequent exposure to the etiologic agent(3). Consequently, considerable effort has been devoted to the development of a vaccine that would be similarly protective. A parenteral whole cell cholera vaccine has been produced, but some no longer regard it as useful, particularly for young children who are at greatest risk from the disease(1).

As for many other infectious diseases, a biological exotoxin (in this case, "cholera toxin" or "CTX") encoded by the genome of the infectious agent and secreted by it, contributes significantly to the ability of the microorganism to colonize the infected

host(4). Moreover, exposure to the toxin causes severe diarrhea and vomiting which result in dehydration, a life-threatening condition of the disease(3,5). These experiences suggest that a vaccine which elicits an immunologic response (e.g., antibodies) sufficient to neutralize the toxin would thus significantly help to prevent or reduce bacterial colonization and attendant symptoms such as diarrhea and vomiting. Thus, substantial effort has been applied toward developing a vaccine containing a non-toxic analog of the toxin, i.e., a "toxoid"(1,3-13). It is known that cholera toxin is a multi-subunit macromolecule consisting of a subunit termed "A", containing a catalytic region called "A1" which ADP-ribosylates G-proteins in target cells, and a "B" oligomer which binds the holotoxin to the target cells(6). Non-toxic analogs of cholera toxin have been produced for purposes of vaccine development by various means. These methods include chemical treatment of the holotoxin or toxin subunits, deletion of the A subunit and use of the remaining B oligomer, and synthesis or isolation of peptide fragments of toxin subunits(1,3-13).

In recent years, efforts have turned toward the development of oral vaccines, with two approaches apparently having received the most attention. One of these approaches is based on the use of killed *V. cholerae* (i.e., chemically- or heat-inactivated), alone, or supplemented with the B oligomer of cholera toxin(1,11,12). This approach has been found to produce incomplete protection, particularly in young children(12). The other approach involves the use of living, but attenuated, strains of *V. cholerae* which fail to produce the A1 subunit of the toxin(13). Vaccines of this kind have provided greater levels of protection, but until recently have also been associated with unacceptable intestinal side-effects.

A recently-developed vaccine based on *V. cholerae* strain CVD 103-HgR, in which the gene encoding the A subunit is omitted, appears to be better tolerated, at least in adults(13). However, to our knowledge, this vaccine has not been tested in children or in large-scale clinical trials.

Recent studies on the nature of cholera toxin have provided insights concerning its structure that may have application in vaccine development based on a recombinant approach. It is known that naturally-ocurring subunit A is synthesized in *V. cholerae* as a preprotein(14), which is subsequently cleaved to proteolytically remove a signal peptide sequence of approximately 2,160 kDa. Further post-translational processing yields an amino-terminal polypeptide of approximately 21,817 kDa (subunit A1) and a carboxyl-terminal polypeptide of approximately 5,398 kDa (subunit A2), which are linked by a disulfide bridge(6,15,16); reduction of the disulfide bond is believed necessary for catalysis of the ADP-ribosyltransferase reaction (6,15,16). Likewise, the B subunit is synthesized as a preprotein which is subsequently cleaved by protease to remove a signal peptide. The genes, or cistronic elements, for the A1, A2 and B subunits of cholera toxin have all been fully sequenced and described in the literature(16).

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1A is the DNA sequence of the cistronic element encoding the A subunit of CTX from the prior art. The single-letter amino acid sequence beneath the DNA sequence indicates the proposed open reading frame for the A polypeptide. Subregions are also indicated, showing the start of the signal peptide (pre-A), A1, two proposed sites for carboxyl-terminal

processing of A1, and the proposed start and termination of A2. It should be noted that the literature provides inconclusive evidence as to the exact location of the carboxyl terminus of A1(16,17).

FIGURE 1B is the DNA sequence of the cistronic element encoding the B subunit of CTX. Initiation and termination codons and proposed cleavage sites are likewise shown. Interestingly, the region of DNA in the operon encoding the termination of A2 and the initiation of B overlap; these two proteins, however, are in different reading frames.

FIGURE 2 shows schematic structures for the preprotein and processed protein forms of the A and B subunits of native CTX and the forms of the recombinant subunits. The "squiggle" at the amino termini of the preprotein species represents the signal peptide which is removed by *V. cholerae*. "M" indicates an amino terminal methionine residue; "(M)" indicates that this is a heterologous (non-native) residue residing at the amino terminus of the mature recombinant CTXA and CTXA1 subunits, and analogs thereof; amino acid sequence data indicates that the heterologous methionine residue is not substantially cleaved from the recombinant polypeptide by cellular methionine amino-peptidase. "S" indicates the sulfur moiety involved in a disulfide linkage between cysteine residues. Other selected amino acids are indicated by their standard single-letter codes, with their position within the polypeptides indicated. Selected restriction enzyme cleavage sites for the encoding DNA sequences are indicated on the encoded polypeptide with their standard three-letter codes. Native ("n") CTXA is believed to be synthesized in *V. cholerae* as a preprotein ("pre-A"), containing an amino-terminal

signal sequence. Post-translational processing results in cleavage of the signal to yield mature CTXA. Perhaps simultaneously, a small portion of the carboxyl terminus is also cleaved proteolytically. The larger A fragment (CTXA1) and the smaller carboxyl-terminal A fragment (CTXA2) are held together after cleavage by a disulfide bridge between the single cysteine residue in each fragment. The literature possesses conflicting reports as to the location of the terminus of CTXA1 (either Arg^{192} or Ser^{194}); CTXA2 is believed to begin with Met^{195}. Native ("n") CTXB is also synthesized with an amino-terminal signal sequence that is subsequently processed by protease. Interestingly, the region of the CTXB cistronic element encoding its amino terminus overlaps with the CTXA cistronic element encoding its carboxyl terminus; the coding sequences, however, are in different reading frames(16). Recombinant ("r") CTXA was synthesized in *E. coli* under control of an optimized expression vector. An oligonucleotide linker (NdeI-XbaI) was used for cloning of the left-hand end of the DNA element, substituting an initiating methionine codon for the signal peptide-encoded sequence. The A2 region was not removed from A1 in the recombinant *E. coli*. A similar left-hand cloning strategy was used for CTXB, except an NdeI-AccI fragment was used to substitute the methionine initiation codon for its signal peptide-encoded sequence. Recombinant CTXA1 was synthesized to mimic native, reduced CTXA1. In this regard, an oligonucleotide linker at the right-hand end was used to substitute a termination codon for the A2 sequence such that A1 terminates at Ser^{194}, one of the two proposed cleavage sites in native CTXA1. Termination at Arg^{192} can also be easily accomplished using the same linker strategy. As previously noted, the amino terminal methionines of the recombinant CTXA and CTXA1

molecules, and their analogs, are not believed to be substantially removed by nascent *E. coli* methionine aminopeptidase.

FIGURE 3 is the SDS-PAGE of native and recombinant CTX subunits. Recombinant CTXA, CTXA1, the $Arg^{7}\rightarrow Lys$ analogs of recombinant CTXA and CTXA1, and recombinant CTXB were synthesized in *E. coli* and inclusion bodies prepared as described in the text. The inclusion body preparations, as well as purified commercial-grade native CTX, CTXA, and CTXB, were solubilized and subjected to SDS-PAGE under reducing conditions. Lane 1, native CTX; lane 2, rCTXA/A7; lane 3, rCTXA $Arg^{7}\rightarrow Lys$ analog (rCTXA/L7); lane 4, rCTXA1/A7; lane 5, rCTXA1 $Arg^{7}\rightarrow Lys$ analog (rCTXA1/L7); lane 6, rCTXB; lane 7, native CTXB; lane 8, native CTXA (only CTXA1 is visualized). Subsequent to electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 and then destained to reveal the stain-retaining polypeptides.

FIGURE 4 is the SDS-PAGE and autoradiographic analysis of rCTXA1 and CTXA1 analog ADP-ribosyltransferase activity. In Panel A, native CTXA, recombinant CTXA1, and various site-specific analogs or preparations of rCTXA1 were subjected to SDS-PAGE and stained with Coomassie Blue. These same preparations were used as enzyme sources to ADP-ribosylate membrane-associated G protein using [^{32}P]NAD under assay conditions described in the text. After the reactions were quenched, the entire reaction mixture from each preparation was subjected to SDS-PAGE, and the gel dried and subjected to autoradiography to visualize proteins that have been covalently modified by addition of [^{32}P]-labeled ADP-ribose. Panel B shows the result of the assays when no G-protein substrate was added,

illustrating the ability of recombinant CTXA1 to autoribosylate; interestingly, analog CTXA1/L7 has lost this reactivity. Panel C shows the ADP-ribosylation of substrate G protein found in human erythrocyte membranes. Addition of this substrate substantially shifts reactivity of the enzyme from itself (autoribosylation) to the target G protein (seen in the autoradiogram as its ribosylated α-subunit). Again, rCTXA1 analog L7 lacks this reactivity.

FIGURE 5 is the SDS-PAGE and autoradiographic analysis of rCTXA and rCTXA analog ADP-ribosyltransferase activities, similar to that shown for rCTXA1 in Figure 4. Because the rCTXA preparation possesses significantly lower activity than rCTXA1 (see Figure 6), presumably because the former still contains the uncleaved A2 "tail" at its carboxyl terminus, these autoradiograms were attained by a longer exposure of the gel (Panel A) to the x-ray film. Panel A is the stained SDS-polyacrylamide gel of the rCTXA proteins; in comparison with Figure 4, Panel A, it is evident that the recombinant expression of these proteins is generally less than that of the companion rCTXA1 proteins. The recombinant CTXA preparation was capable of autoribosylation (Panel B) and of ADP-ribosylating the G protein substrate in human erythrocyte membranes (Panel C); these activities are substantially diminished in comparison with rCTXA1. Nevertheless, the CTXA preparations exhibit the same general pattern of inactivation as do their CTXA1 counterparts. Again, the L7 analog (Arg7→Lys) is devoid of ADP-ribosylating activity.

FIGURE 6 is the SDS-PAGE and autoradiographic comparison of the ADP-ribosyltransferase activity of rCTXA and rCTXA1/L7 with that of rCTXA1 and rCTXA1/L7.

Panel A is the reactivity without added substrate and Panel B is with human erythrocyte membranes added as substrate. The lanes contain: lane 1) blank (no sample added to reaction); lane 2) native CTXA without urea treatment; lane 3) native CTXA with urea treatment; lane 4) rCTXA; lane 5) rCTXA/L7; lane 6) rCTXA/L7 plus native CTXA; lane 7) rCTXA1; lane 8) rCTXA/L7; lane 9) rCTXA1/L7 plus native CTXA. This experiment demonstrates that the rCTXA preparation is much less active than rCTXA1 for ADP-ribosylation of G proteins (compare lanes 4 and 7), yet exhibits substantial autoribosylating activity. Confirming the data shown in Figures 4 and 5, substitution of lysine for arginine-7 in rCTXA and rCTXA1 abolishes their ribosylating activities, both for autocatalysis and for G protein. Retention of activity by native CTXA when added to the analog preparations (lanes 6 and 9) additionally illustrates that it is not a contaminant of the recombinant preparations that suppress this activity.

FIGURE 7 illustrates the ADP-ribosylation of H27 fibroblast and erythrocyte membranes by CTXA and CTXA1 analogs. Naturally-occurring CTXA or recombinant CTXA1 analogs were incubated with [^{32}P]NAD and either human erythrocyte or H27 fibroblast membranes. After incubation, the mixtures were precipitated, centrifuged, and the resulting pellets subjected to SDS-PAGE. The gels were stained with Coomassie Blue, dried, and subsequently exposed to x-ray film to produce autoradiograms. B, no CTXA or CTXA1 analog added; A, naturally-occurring CTXA, A+u, naturally-occurring CTXA treated with urea; rA1, recombinant CTXA1 with no residue substitutions; RBC, human erythrocyte membranes.

FIGURE 8 illustrates the ADP-ribosylation of H27 fibroblast and membranes by CTXA and CTXA1 analogs. Naturally-occurring CTXA or recombinant CTXA1 analogs were incubated with [^{32}P]NAD in the presence of either human erythrocyte membranes, H27 fibroblast membranes, or no added substrate-containing membranes. After incubation, the mixtures were precipitated, centrifuged, and the resulting pellets were subjected to SDS-PAGE. The gels were stained with Coomassie blue, washed and dried. The upper left panel is a photograph of a stained gel of samples incubated in the absence of substrate-containing membranes; the upper right panel is an autoradiogram of this gel. The lower left and right panels are autoradiograms of gels of samples incubated with erythrocyte and H27 membranes, respectively. B, no CTXA or CTXA1 analog added; A, naturally-occurring CTXA; A + u, naturally-occurring CTXA treated with urea; rA1, recombinant CTXA1 with no residue substitutions; RBC, human erythrocyte membranes.

SUMMARY OF THE INVENTION

The present invention provides a recombinant DNA molecule, at least a portion of which encodes an analog of the catalytic subunit of cholera toxin having reduced enzymatic activity, such activity generally accepted to be associated with vaccine reactogenicity. More specifically, site specific mutagenesis, as described herein, results in analogs of the A and A1 subunits which, compared to the native toxin counterparts, exhibit a significant reduction in catalytic function as measured by ADP-ribosyltransferase activity.

The term "catalytic subunit of cholera toxin" used in this disclosure refers to both the

A region of cholera toxin and the A1 subregion, as depicted in Figs. 1A and 2. These regions of the cholera toxin macromolecule are known to possess ADP-ribosyltransferase catalytic activity(6). This enzyme is a complex of two sub-activities: an NAD glycohydrolase activity which cleaves NAD into nicotinamide and ADP-ribose, and a transferase activity which transfers the ADP-ribose to the G protein substrate. Measurements of the ADP-ribosyltransferase activity in this disclosure represent a summation of both activities. The present invention comprehends mutagenesized versions of these A and A1 polypeptides, and analogs or derivatives of such polypeptides, which in their native forms are sources of catalytic activity within the cholera toxin multimer.

The genetically-engineered analogs of cholera toxin, which are a product of this invention, provide recombinant DNA-derived materials suitable for use in vaccines for the prevention of cholera disease. The A and A1 subunit analogs can be used alone or in combination with B oligomer in a toxoid-based vaccine, or phenotypically expressed by variants of *V. cholerae*, or phenotypically expressed under the genetic control of other immunizing vectors. It should be noted that the analog A and A1 subunits of this invention are utilizable by themselves as antigenic agents in a vaccine because they may contain important protective epitopes. However, the use of these analogs in association with B subunits may be more desirable. The B oligomer contains neutralizing epitopes useful for eliciting immunoprotection(1,3,5). Association of the A subunit with the B oligomer may lead to a more effective immunogenic response against the B oligomer. The B oligomer can be purified from *V. cholerae* or, alternatively, can be derived recombinantly in a manner similar to the A and A1 subunits by expression in

E. coli or other recombinant hosts, including other bacterial organisms (e.g., *Salmonella typhimurium* or *typhi*, *Bacillus sp.*), yeast (e.g., *S. cerevisiae)*, and viruses (e.g., vaccinia and adenoviruses).

Mutagenesis in accordance with this description enables production of mutants varying in diminished catalytic activity, ranging from variants which exhibit attenuated activity to those which are essentially free of such activity (i.e., less than 5%). This flexibility in approach is desirable because attenuation, rather than elimination, of catalytic activity may be helpful in providing a greater degree of and/or longer-lasting, protective response. Moreover, because of their diminished enzymatic activity, the analog subunits provided by this invention are expected to be less reactogenic.

DETAILED DESCRIPTION

The present invention provides high-level, direct recombinant expression of all CTX subunits necessary for vaccine production. Further, catalytic subunit analogs provide biological activity that is reduced in, or essentially free of, ADP-ribosyltransferase catalytic activity. The present analogs used alone, or in combination with B oligomer of the toxin (whether derived from natural sources or by recombinant means), can provide products that are useful in a vaccine and greatly reduce the likelihood of side-effects generally accepted to be associated with the catalytic activity in the native toxin. The toxin analogs of the present invention can be formulated into vaccine compositions or used in combination with other immunogenic agents in a multi-component vaccine.

The individual cistronic elements, or portions thereof, encoding the A and B subunits of *V. cholerae* toxin were subcloned and directly expressed individually in a recombinant host cell system (i.e., *E. coli*). In the absence of a native signal peptide (substituted with a methionine to initiate translation), high levels of expression, in the range of 2% to 80% of total cell protein, were obtained. The fermentation of expressor cells resulted in mature species of rCTXA, rCTXA1 and rCTXB, as shown in Fig. 3. It should be noted that rCTXA is not processed to rCTXA1 and rCTXA2 in *E. coli*, presumably due to the absence of the specific enzyme or a failure of rCTXA to be compartmentalized with this enzyme. Thus, rCTXA possesses the A1 sequence covalently linked to the A2 sequence.

Amino acid analysis of selected recombinant molecules demonstrated that the heterologous (non-native) methionyl residue is not substantially removed from the various rCTX and rCTXA1 subunit species by cellular methionine aminopeptidase; thus, these are also methionyl-mature analogs. All of the recombinant proteins were recovered as inclusion bodies from lysed cells. The subunits were found to have migration patterns in reducing SDS-PAGE essentially identical to authentic native subunits, with the exception of rCTXA which is not processed in *E. coli* to result in cleavage of the A2 region from A1. As shown in Fig. 3, high-level recombinant expression of subunits CTXA, CTXA1 and CTXB in *E. coli* was achieved by direct, non-fusion means.

Although alternative methods and materials can be used in the practice of the present invention, the preferred methods and materials are described

below. All references cited hereunder are incorporated herein by reference.

MATERIALS AND METHODS FOR RECOMBINANT EXPRESSION OF CTXA, CTXA1 AND CTXB SUBUNITS

Materials. DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA), Bethesda Research Laboratories (Gaithersburg, MD), Boehringer Mannheim Biochemicals, (Indianapolis, IN), and International Biotechnologies, Inc. (New Haven, CT); enzymes were used according to manufacturer recommendations. All chemicals and biochemicals were analytical reagent grade. Purified, naturally-occurring cholera toxin and toxin subunits were purchased from Sigma Chemical Company (St. Louis, MO) and List Biologicals (Campbell, CA). Synthetic oligonucleotides were synthesized based on methods developed from the chemical procedure of Matteucci and Caruthers(18).

Plasmids and Bacterial Strains. Plasmids pRIT10810 and pRIT10841, (ATCC 39051 and ATCC 39053, respectively), containing the portions of the CTX operon, were obtained from the American Type Culture Collection, Rockville, MD. Expression plasmids pCFM1036, pCFM1146 and pCFM1156 were derived at Amgen.

A description of the expression vector system used herein is described in United States Patent No. 4,710,473 (Morris), which is incorporated herein by reference. Such plasmids contain an inducible promoter, a synthetic ribosome binding site, a cloning cluster, plasmid origin of replication, a transcription terminator, genes for regulating plasmid copy number, and a Kanamycin resistance gene. The derived plasmids differ from each other in a number of respects. The plasmid

pCFM1036 can be derived from pCFM836 (see U.S. 4,710,473) by substituting the DNA sequence between the unique AstII and EcoRI restriction sites containing the synthetic P_L promoter with the following oligonucleotide:

AatII EcoRI

5′-CATCGATTCTAG-3′
3′-TGCAGTAGCTAAGATCTTAA-5′

This plasmid contains no inducible promoter preceding the restriction cluster. The plasmid pCFM1146 can be derived from pCFM836 by substituting the small DNA sequence between the unique ClaI and XbaI restriction sites with the following oligonucleotide:

ClaI XbaI

5′-CGATTTGATT-3′
3′-TAAACTAAGATC-5′

and by destroying the two endogenous NdeI restriction sites by end-filling with T4 polymerase enzyme followed by blunt-end ligation. The plasmid contains no synthetic ribosome binding site immediately preceding the restriction cluster. The plasmid pCFM1156 can be derived from pCFM1146 by substitution of the small DNA sequence between the unique XbaI and KpnI restriction sites with the following oligonuceotide which installs an optimized synthetic ribosome binding site:

XbaI KpnI

5′-CTAGAAGGAAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC-3′
3′-TTCCTTCCTTATTGTATACCAATTGCGCAACCTTAAGC-5′

Plasmids pBR322, pUC18, pUC19, and phage M13mp18 and M13mp19 DNA were purchased from Bethesda Research Laboratories. *E. coli* FM5 cells were derived at Amgen Inc., Thousand Oaks, CA from *E. coli* K-12 strain(19) from C.F. Morris and contain the integrated lambda phage repressor gene, CI_{857}(20). Construction of the individual subunit expression plasmids is described herein. Vector production, cell transformation, and colony selection were performed by standard methods(21).

Analytical Procedures. DNA sequencing was done by modification of the primer-extension, chain-termination method(22,23). Protein sequence analyses were performed by automated Edman degradation in an ABI 470A gas-phase microsequenator(24,25) and by standard enzymatic means, the latter to obtain carboxyl-terminal sequences of selected-proteins. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli(26), and elution of polypeptides from polyacrylamide gels was similar to the method of Hunkapiller *et al.*(27). The ratio of recombinant protein to total cellular protein or total inclusion body protein was assessed by SDS-PAGE of whole-cell lysates followed by staining with Coomassie Brilliant Blue R250 and subsequent gel scanning by integrative densitometry.

Assays for the measurement of ADP-ribosyltransferase catalytic activity were done as follows: Native CTXA and recombinant subunits were incubated in a solubilization buffer of 8 M urea, 25 mM sodium phosphate (pH 7.0) and 10 mM dithiothreitol (DTT) for one hour at 37°C and centrifuged at 10,000 rpm for 15 minutes without refrigeration. The additions to the solubilization buffer were adjusted to yield 1 µg of native or recombinant A1 per 4 µL, which

was then added to 60 µL of a reaction mixture (see below) and incubated for one hour on ice.

Reaction Mixture

Reagent*:	(final)/60 µl	(final)/100 µl
Na_xPO_4, pH 7.0, 1 M	416 mM	250 mM
DTT, 100 mM	5 mM	3 mM
GTP, 10 mM	167 µM	100 µM
Thymidine, 100 mM	17 mM	10 mM
$MgCl_2$, 1 M	5 mM	3 mM
[^{32}P]-NAD	2.5 µCi	2.5 µCi
NAD, 2500 µM	50 µM	30 µM

*The reagents were obtained from commercial sources. Naturally-occurring CTXA was acquired from List Laboratories. As a control, native CTXA was also assayed by incubation in the same buffer as above, but without urea, for 15 minutes at 37°C, then kept on ice until assayed for ADP-ribosyltransferase activity.

Thirty-six µL of water or a buffer containing human erythrocyte membranes(28) were added to yield a final volume of 100 µL for each sample and the samples incubated at 30°C. After 30 minutes, the reaction was terminated by adding 50 µL of 5 mM NAD and 0.03% sodium deoxycholate to each sample and the reaction mixture chilled on ice for 10 minutes. Fifty µL of 40% trichloroacetic acid (TCA) were then added, the samples placed on ice for at least 15 minutes; 2 mL of water were subsequently added to each sample, and the precipitated protein pelleted by centrifugation. The supernatants were removed and the pelleted protein was frozen. On the following day, the pelleted protein was

subjected to SDS-PAGE(26,29). The gel was stained with Coomassie Brilliant Blue, destained, dried and subjected to autoradiography to measure the content of covalently linked [^{32}P]-labeled ADP-ribose in the proteins of the various bands. An approximation of the specific activities of the recombinant CTXA1 and recombinant analog CTXA1 proteins (relative to the activity of native CTXA1) was obtained by densitometric scanning of the gels and autoradiograms. The stained gels were scanned to approximate the amount of individual protein added to each reaction mixture. The autoradiograms were scanned to estimate the amount of [^{32}P]ADP-ribose transferred to the G protein substrate as a function of the density of the autoradiographic image.

Construction of Expression Plasmids. All expression plasmids were constructed from a series of *E. coli* generalized expression vectors differing as described previously. The individual cholera toxin subunit gene segments were isolated using the restriction sites shown in Figs. 1 and 2. The upstream restriction site was just inside the codon for the amino-terminal residue of the mature, processed form of the subunit (i.e., without the signal sequence). For purposes of recombinant expression in *E. coli*, the portion of the CTX genes encoding their native signal peptides were deleted and substituted instead by a methionine initiation codon, for expression of the "methionyl-mature" form of the subunit analogs. Synthetic oligonucleotide linkers were employed to effect insertion of the gene segments into the expression plasmids at an optimal distance downstream of the synthetic promoter and ribosome binding site. The upstream linkers restored the reading frame of each gene back to the first codon of

the mature amino terminus; the oligonucleotides included a methionyl initiation codon.

Following transformation of *E. coli* FM5 cells with the various plasmid constructs and plating on Kanamycin-containing agar, appropriate numbers of colonies were selected, replica-plated, grown as small liquid cultures ("minipreps"), and induced at 42°C for 4 hours. The minipreps were then screened by light microscopy for the presence of inclusion bodies in the bacterial cells. Preparations exhibiting apparent inclusions were identified and matching colonies from the replica plates subjected to flask-scale laboratory fermentation at the induction temperature. Samples were removed from fermentation at various times post-induction and examined for the appearance of the appropriate CTX subunit by SDS-PAGE followed by Coomassie Brilliant Blue-staining. The structure of the plasmid from each expression clone was confirmed by restriction mapping of the isolated plasmid and verified by DNA sequencing of junction regions.

Expression of Recombinant CTX, CTXA1 and CTXB. When *E. coli* cells containing, separately, the CTXA expression plasmid (pCTXA/A7/1156), the CTXA1 expression plasmid (pCTXA1/A7/1156), and the pCTXB expression plasmid (pCTXB/1156) were fermented at 37°C and 42°C, they produced major intracellular proteins (Figure 3) of approximately 27,215 daltons, 21,817 daltons and 11,600 daltons, respectively; recombinant CTXA1 and CTXB comigrated with authentic (native) CTXA1 and CTXB, respectively, in SDS-PAGE. Our recombinant CTXA has no native counterpart, since natural CTXA is cleaved to CTXA1 and CTXA2 by *V. cholerae* protease at some point before secretion from the organism; A1 and A2 are held together by a disulfide bond that is reduced by the buffers used in SDS-PAGE. Partial amino acid sequence analysis established that recombinant

polypeptide CTXA1/A7 and CTXA1/L7 (see description below) had the amino terminal sequence predicted for the native CTXA1 subunit, but that the heterologous initiating methionine residue is not substantially removed.

Properties of Recombinant CTX Subunits. Very little, if any, of the CTX subunits appear to be secreted from the *E. coli* cells. The bulk of each subunit was found in the form of inclusion bodies and constituted 2% to 80% of total cellular protein. Cell lysis by French press and low speed centrifugation resulted in pellet fractions that contained up to 80% of their protein as the individual subunits. All the rCTX subunits were detectable in gels stained with Coomassie Brilliant Blue (Figure 3).

CTXA AND CTXA1 ANALOGS

Using techniques of protein engineering and site-specific mutagenesis(19,30), CTXA and CTXA1 analogs were made. From those analogs made and tested by the time of this submission, it was found that mutagenesis of the amino acid residues at positions arginine-7, histidine-44, histidine-70, glutamic acid-112, and aspartic acid-9, and truncation of the carboxyl terminus (at tryptophan-179 of the mature native CTXA sequence) resulted in diminished or essentially no ADP-ribosyltransferase activity.

Construction of the CTXA Expression Plasmid. Plasmid pRIT10841 (ATCC 39053) was cleaved with restriction enzymes XbaI and ClaI and a 552-bp DNA fragment was isolated by gel electrophoresis which contained the left-hand end of the CTXA gene to the region encoding the protease-sensitive portion that results in CTXA cleavage to CTXA1 and CTXA2. Plasmid pRIT10810 (ATCC 39051) was cleaved with restriction

enzymes ClaI and HindII (the latter an isoschizomer of HincII) and a 368-bp DNA fragment was isolated that encoded a portion of the CTXA subunit from the protease-sensitive site (encoded at the ClaI site) (16,17) through the CTXA2 region, past the termination codon of CTXA, and into the alternative open reading frame of the CTXB subunit.

A synthetic oligonucleotide linker was prepared to reconstitute the open reading frame of CTXA from the site encoding the first amino acid of the mature protein sequence (asparagine) to the XbaI site. This linker possessed NdeI cohesiveness at its left-hand end in order to generate a methionine initiation codon that would substitute for the sequence encoding the signal peptide and to facilitate insertion of the gene construction into the expression vector; the right-hand end of the linker possessed an XbaI overlap. This linker possessed the sequence:

5'-TATGAATGATGATAAGTTATATCGGGCAGATT-3'
3'-ACTTACTACTATTCAATATAGCCCGTCTAAGATC-5'

Plasmid pUC19 was digested with NdeI and XbaI and the linker above inserted. After ligation and transformation, a pUC plasmid named p2A/pUC19 was isolated that contained the linker sequence in place of the normal pUC19 NdeI-XbaI sequence.

Plasmid p2A/pUC19 was digested with XbaI and HincII. The large fragment from this digestion was ligated together with the 552-bp XbaI-ClaI DNA fragment containing the left-hand end of the CTXA gene and the 368-bp ClaI-HindII DNA fragment containing the right-hand end of the CTXA gene (past the termination codon and into the alternative open reading frame of the CTXB subunit). This produced a new plasmid containing the

entire mature CTXA gene; this plasmid was called pCTXA/A7/pUC19.

The *E. coli* expression plasmid pCFM1156 was digested with NdeI and HindIII to remove this small portion of its cloning cluster. Plasmid pCTXA/A7/pUC19 was also digested with NdeI and HindIII, and a DNA fragment (772-bp) containing the entire region of the CTXA gene was isolated. This fragment was subsequently ligated into the digested pCFM1156 plasmid to produce the CTXA expression plasmid pCTXA/A7/1156. This NdeI-NdeI fragment could be inserted into pCFM1156 in either of two orientations, only one of which would produce an open reading frame giving rise to a large protein when expressed. This clone was selected (by analysis of induced clones by SDS-PAGE to identify the recombinant CTXA protein) and the proper orientation confirmed by DNA sequencing at the upstream NdeI junction region.

Construction of the CTXB Expression Plasmid.

Plasmid pRIT10810 (ATCC 39051) was digested with ClaI and BstXI and a 538-bp DNA fragment was isolated; this contained the the A2 coding region of CTXA, the entire CTXB coding region, and a short DNA sequence to the right of the termination codon of CTXB.

A synthetic oligonucleotide linker was prepared that permitted the cloning of the right-hand end of the DNA sequence above into pUC19. This linker possessed BstX1 and HindIII cohesive ends and had the sequence:

5'-GTGGAATTCGGTACCATGGA-3'
3'-GAGTCACCTTAAGCCATGGTACCTTCGAA-5'

Plasmid pUC19 was digested with HindIII and AccI (the latter generating a cohesive end compatible with that generated by ClaI). The large pUC19 fragment

was ligated with the 538-bp ClaI-BstXI DNA fragment containing the CTXB and the BstXI-HindIII linker to produce a plasmid called pCTXB/pUC19. This plasmid was then digested with HindIII and SspI (the latter just inside the initiation codon for CTXB and downstream from the ClaI site) to isolate a 345-bP SspI-HindIII fragment.

A synthetic oligonucleotide linker was prepared that possessed NdeI and SspI cohesive ends and the sequence:

5'-TATGACACCTCAAAAT-3'
3'-ACTGTGGAGTTTTA-5'

Plasmid pCFM1156 was digested with NdeI and HindIII to remove this portion of its cloning cluster. The large pCFM1156 DNA fragment was then ligated with the 345-bp SspI-HindIII fragment containing a portion of the CTXB gene and the NdeI-SspI linker that restored its left-hand coding region and insinuated a methionine codon at the left of this coding region to initiate protein synthesis. The subsequent expression plasmid, containing the entire CTXB gene with a methionine initiation codon, was called pCTXB/1156.

Linker Mutagenesis. An oligonucleotide linker called L7 was synthesized to substitute a lysine codon for that of arginine-7 in CTXA. The sequence of this linker, with NdeI and XbaI cohesive ends, is shown in Table 1. The L7 linker was cloned into the NdeI-XbaI site of pUC19 to produce a plasmid called pL7/pUC19. Plasmid pL7/pUC19 was then digested with XbaI and HindIII to remove this portion of the pUC19 cloning cluster and replaced through ligation with the 552-bp XbaI-ClaI DNA fragment containing the left-hand end of the CTXA gene (see above) and the 368-bp ClaI-HindII DNA fragment containing the right-hand end of this gene

(see above). This plasmid, called pCTXA/L7/pUC19, was digested with NdeI, and a 772-bp DNA fragment was isolated that possessed the entire mature CTXA gene with a substitution of the arginine-7 codon by a lysine codon. Plasmid pCFM1156 was digested with NdeI and ligated with the NdeI DNA fragment from pCTXA/L7/pUC19. This ligation produced a plasmid called pCTXA/L7/1156 for expression of the mature form of an $Arg^7 \rightarrow Lys$ analog of CTXA in *E. coli*. As with the case of pCTXA/A7/1156 (above), it was necessary to select a clone containing this plasmid with the DNA insert in the proper open reading frame for synthesis of rCTXA/L7.

Oligonucleotide linkers 1E and 1F were synthesized to individually substitute, respectively, a phenylalanine codon for that of tyrosine-6 and a glutamate codon for that of aspartate-9. These linkers possessed NdeI and XbaI cohesive ends and had the sequences shown in Table 1. Plasmid pCTXA/A7/pUC19 (see above) was digested with XbaI and HindIII, and a 938-bp DNA fragment containing the right-hand portion of the CTXA gene was isolated. Plasmid pCFM1156 was digested with NdeI and HindIII to remove this short region of its cloning cluster. This segment was replaced by ligation with the NdeI-XbaI linker containing either the $Tyr^6 \rightarrow Phe$ or the $Asp^9 \rightarrow Glu$ codon mutation (linkers 1E and 1F, respectively) and the 938-bp DNA fragment of the CTXA gene. This produced two plasmids, pCTXA/1E/1156 and pCTXA/1F/1156, for expression of the mature forms of the CTXA analogs $Tyr^6 \rightarrow Phe$ and $Asp^9 \rightarrow Glu$, respectively, in *E. coli*.

The substitutions of sequences encoding mutations of glutamine for proline-185 and alanine for cysteine-187 resulted in CTXA gene fragments encoding only the CTXA1 portion of the CTXA subunit (see below for construction of the native-sequence CTXA1 gene and

the L7, 1E, and 1F substitution analogs of CTXA1 from the CTXA gene and its substitution analogs, respectively). Oligonucleotide linkers 1G and 1H were synthesized to individually substitute, respectively, glutamine for proline-185 and alanine for cysteine-187. These linkers had DsaI and HindIII cohesive ends and possessed the sequences shown in Table 1. To effect the construction of the expression plasmids encoding the analog proteins, a 537-bp NdeI-DsaI DNA fragment was isolated from plasmid pCTXA/A7/pUC19. Plasmid pCFM1156 was then digested with NdeI and HindIII to remove this short segment of its cloning cluster. This segment was replaced by ligation with the 537-bp DNA fragment from pCTXA/A7/pUC19 and either 1G or 1H synthetic oligonucleotides. The linkers, in addition to encoding the specific amino acid substitutions, eliminate from the CTXA gene that portion encoding the A2 region of the CTXA subunit; thus, these mutations are exclusively in CTXA1 versions of the subunit. The resulting plasmids for expression of the $Pro^{185}\rightarrow Gln$ and $Cys^{187}\rightarrow Ala$ analogs of CTXA1 were called pCTXA1/1G/1156 and pCTXA1/1H/1156, respectively.

A plasmid expressing a carboxyl-terminal truncated version of CTXA1 terminating at Trp^{179} was constructed. This was accomplished by first digesting plasmid pCFM1156 with NdeI and HindIII to remove this short DNA fragment. Into this site in pCFM1156 was ligated the 537-bp NdeI-DsaI fragment from pCTXA/A7/PUC19 (see above) and a synthetic DNA fragment with DsaI and HindIII cohesive ends, and having the sequence:

5′-CGTGGTAATGATAGA-3′
3′-CATTACTATCTTCGA-5′

This plasmid, for expression of CTXA1 truncated at Trp^{179}, was called pCTXA1/T1/1156.

Mutagenesis By Site-directed Priming.

Mutagenesis by site-directed priming was accomplished with kits of the "Altered Sites™ *in vitro* Mutagenesis System" purchased from Promega Corporation (Madison, WI); details of the experimental protocols for this procedure are contained in the technical manual available from Promega Corporation (printed 1/90).

To facilitate mutagenesis, a 938-bp XbaI-HindIII DNA fragment encoding a portion of the CTXA subunit was isolated from plasmid pCTXA/A7/pUC19 (see above). This fragment was cloned into the pSELECT1 phagemid vector from Promega. After packaging with helper phage, this vector contained a negative-sense copy of the CTXA fragment. A series of single-stranded, positive-sense DNA primers were synthesized to effect mutagenesis; the sequences of these primers (1B, 1C, 1D, and 1I) are shown in Table 1. These primers were individually annealed with the single-stranded phagemid containing the CTXA gene fragment; double-stranded phagemids were subsequently produced which contained the gene fragment and the individual codon substitutions encoded by the primers.

For preparation of plasmids capable of expressing the CTXA and CTXA1 subunit analogs containing a lysine substitution for arginine-146, a 207-bp BstXI-ClaI DNA fragment was isolated from the double-stranded phagemid containing the $Arg^{146}\rightarrow Lys$ codon mutation (1I). A 375-bp NdeI-BstXI DNA fragment and a 386-bp ClaI-HindIII fragment (for the CTXA version) containing a portion of the CTXA gene were isolated from plasmid pCTXA/A7/pUC19. Plasmid pCFM1156 was digested with NdeI and HindIII to remove this short portion of its cloning cluster. For construction of the CTXA version of the $Arg^{146}\rightarrow Lys$ mutation, the

digested pCFM1156 plasmid was ligated with the 375-bp NdeI-BstXI fragment from pCTXA/A7/pUC19, the 209-bp BstXI-ClaI fragment from the double-stranded phagemid, and the 386-bp ClaI-HindIII DNA fragment from pCTXA/A7/pUC19. This resulted in a plasmid called pCTXA/1I/1156 for expression of the $Arg^{146}\rightarrow Lys$ analog of the CTXA subunit in *E. coli*. For construction of this mutation in the CTXA1 version of the subunit, the digested pCFM1156 plasmid was ligated with the 375-bp NdeI-BstXI fragment from pCTXA/A7/pUC19, the 209-bp BstXI-ClaI fragment isolated from the double-stranded phagemid, and a synthetic oligonucleotide linker that replaces a region of CTXA encoding the A2 portion of CTXA with a DNA sequence encoding the end of the A1 region and including a codon that terminates polypeptide synthesis at the end of CTXA1. This linker possessed ClaI and HindIII cohesive ends and had the sequence:

5′CGTAATAGGCGGCCGCA-3′
3′-ATTATCCGCCGGCGTCGA-5′

The resultant plasmid for expression of the $Arg^{146}\rightarrow Lys$ analog of CTXA1 in *E. coli* was called pCTXA1/1I/1156.

Preparation of plasmids capable of expressing individual analogs of CTXA containing the substitutions of $His^{44}\rightarrow Asn$, $His^{70}\rightarrow Asn$, or $Glu^{112}\rightarrow Gln$ was facilitated with primers (1B, 1C, and 1D, respectively) having the sequences shown in Table 1. After annealing of the primers individually to the pSELECT1 phagemid containing the 938-bp XbaI-HindIII CTXA fragment from pCTXA/A7/pUC19 (see above) and recovering double-stranded plasmid, the regions containing the site-specific mutations were excised from the plasmid by digesting with XbaI and HindIII,

and recovering a 938-bp DNA fragment in each case. Plasmid p2A/pUC19 (containing an NdeI-XbaI linker encoding the left-hand end of the mature CTXA; see above) was digested with XbaI and HindIII to remove this short region of the pUC19 cloning cluster to the right of the linker insert; this region was replaced by ligation with the 938-bp XbaI-HindIII fragment from the plasmid containing a single codon replacement. This series of pUC-derived plasmids were called pCTXA/1B/pUC19, pCTXA/1C/pUC19, and pCTXA/1D/pUC19, depending upon the codon replacement they contained. A DNA fragment containing the codon replacement was subsequently excised from each of these plasmids. Plasmid CTXA/A7/pUC19 was digested with BstXI and HindIII and a 593-bp DNA fragment was isolated. Plasmid pCFM1156 was digested with NdeI and HindIII to remove this short region of its cloning cluster, as described earlier, and this replaced by ligation with the individual CTXA analog gene inserts recovered from the pUC transition plasmids above and the 593-bp BstXI-HindIII DNA fragment from pCTX/A7/pUC19. When isolated, these new plasmids for expression of the site-specific analogs $His^{44}\rightarrow Asn$, $His^{70}\rightarrow Asn$, and $Glu^{112}\rightarrow Gln$ of CTXA in *E. coli* were called pCTXA/1B/1156, pCTXA/1C/1156, and pCTXA/1D/1156, respectively.

Conversion of CTXA and CTXA Analog Genes to CTXA1 and CTXA1 Analog Genes. With the exception of the plasmid containing the 1I codon substitution (pCTXA1/1I/1156), which was constructed during the mutagenesis process to lack the A2-encoding region, it was useful to convert the CTXA gene-containing and selected individual analog gene-containing expression plasmids to CTXA1 expression plasmids in order to express the A1 truncated version of CTXA that mimicked

the native species of this subunit in reduced holotoxin preparations. To perform this conversion, it was necessary to delete a portion of the gene sequence of the CTXA gene (and the analog genes) to the right of the unique ClaI site. Although the actual site of polypeptide cleavage between the A1 and A2 regions has not been resolved in the prior art literature(16,17), it was decided to initially establish the carboxyl terminus of A1 at serine-194; it should be noted, however, that establishing the terminus at arginine-192 (the other terminus proposed in the literature) is a simple matter of inserting a new linker to substitute a termination codon immediately to the right of the arginine-192 codon.

For our purposes, each of the analog CTXA sequences (and the native CTXA sequence) we wished to convert to CTXA1 versions were excised from their pUC19 transition plasmids (*i.e.*, pCTXA/A7/pUC19, pCTXA/1B/pUC19, pCTXA/1C/pUC19, pCTXA/1D/pUC19, pCTXA/1E/pUC19, pCTXA/1F/pUC19, pCTXA/1G/pUC19, pCTXA/1H/pUC19) with restriction enzymes NdeI (at the sequence encoding the methionine initiation codon) and ClaI (at the site chosen for addition of a termination codon immediately to the right of the serine-194 codon); this DNA fragment was 585-bp in each case. For purposes of substituting a termination codon for the A2-encoding region and subsequent ligation of the gene segments into plasmid pCFM1156, an oligonucleotide linker was synthesized to possess ClaI and HindIII cohesive ends and had the following sequence:

5'-CGTAATAGGCGGCCGCA-3'
3'-ATTATCCGCCGGCGTTCGA-5'

Plasmid pCFM1156 was digested with NdeI and HindIII to remove this portion of its cloning cluster;

this region was replaced by ligation with the ClaI-HindIII linker and with an individual 585-bp DNA fragment from one of the pUC transition plasmids described above. Isolation of plasmid DNA following these ligations resulted in a series of plasmids capable of expressing CTXA1 and CTXA1 analog polypeptides in *E. coli;* plasmids prepared in this manner included pCTXA1/1B/1156, pCTXA1/1C/1156, pCTXA1/1D/1156, pCTXA1/1E/1156, and pCTXA1/1F/1156,.

Expression and Analysis of CTXA and Recombinant Analogs. Following preparation, each plasmid was used to transform a separate preparation of fresh, competent FM5 cells. Transformants were picked, grown as minipreps, induced to produce recombinant protein, and inclusion body-positive samples identified by light microscopy. These samples were fermented at a larger scale (≥ 1 liter) at the induction temperature to prepare greater amounts of each recombinant analog protein. Isolated cell pastes were lysed in a French press after resuspension in distilled H_2O with 1 mM DTT. Inclusion bodies were isolated from these lysates by simple low-speed centrifugation. These inclusion-body protein preparations contained as little as 2% and as much as 80% of the recombinant proteins. The samples were assessed for ADP-ribosyltransferase activity as previously described. The results obtained are shown in Figs. 4, 5, and 6 and in Table 2.

TABLE 1
CONSTRUCTION OF S1 ANALOGS

*	MUTATION	TECHNIQUE	OLIGONUCLEOTIDE SEQUENCE
L7	ARG7->Lys	Linker Insertion	5′-TATGAATGATGATAAGTTATATAAGGCAGATT-3′ 3′-ACTTACTACTATTCAATATATTCCGTCTAAGATC-5′
1B	His44->Asn	Site-directed Priming	5′-CCTTTATGATAACGCAAGAGGAA-3′
1C	His70->Asn	Site-directed Priming	5′GAGAAGTGCCAACTTAGTGGGTC-3′
1D	Glu112->Gln	Site-directed Priming	5′-AGATGAACAACAGGTTTCTGCTT-3′
1E	Tyr6->Phe	Linker Insertion	5′-TATGAATGATGATAAGTTATTCCGGGCAGATT-3′ 3′-ACTTACTACTATTCAATAAGGCCCGTCTAAGATC-5′
IF	Asp9->Glu	Linker Insertion	5′-TATGAATGATGATAAGTTATATCGGGCAGAAT-3′ 3′-ACTTACTACTATTCAATATAGCCCGTCTTAGATC-5′
1G	Pro185->Gln	Linker Insertion	5′-CGTGGATTCATCATGCACCGCAGGGTTGTGGGAATGCTCCAAGATCATCGTAGA-3′ 3′-CTAAGTAGTACGTGGCGTCCCAACACCCTTACGAGGTTCTAGTAGCATCTTCGA-5′
1H	Cys187->Ala	Linker Insertion	5′-CGTGGATTCATCATGCACCGCCGGGTGCAGGGAATGCTCCAAGATCATCGTAGA-3′ 3′-CTAAGTAGTACGTGGCGGCCCACGTCCCTTACGAGGTTCTAGTAGCATCTTCGA-5′
1I	Arg146->Lys	Site-directed Priming	5′-GGGGCTACAAGGATAGATAT-3′
T1	COOH Truncation @ Trp179	Linker Insertion	5′-CGTGGTAATGATAGA-3′ 3′-CATTACTATCTTCGA-5′

*Designation

TABLE 2

ADP-RIBOSYLTRANSFERASE ACTIVITIES OF RECOMBINANT CTXA1 ANALOGS[1]

CTX MOLECULE	MUTATION	PROTEIN ADDED TO ASSAY(mG)[2]	SPECIFIC ACTIVTY FOR HEM G. PROTEIN[3]
Commercial CTXA1 (without urea)	none	1.00	1.00
Commercial CTXA1 (with urea)	none	1.11	0.53
rCTXA1/A7	none	1.56	0.56
rCTXA1/L7	Arg7->Lys	1.46	0
rCTXA1/1B	His44->Asn	1.47	0
rCTXA1/1C	His70->Asn	1.51	0.05
rCTXA1/1D	Glu112->Gln	1.65	0
rCTXA1/1E	Tyr6->Phe	1.04	1.01
rCTXA1/1F	Asp9->Glu	0.91	0.10
rCTXA1/1G	Pro185->Gln	1.23	0.81
rCTXA1/1H	Cys187->Ala	1.14	0.83
rCTXA1/1I	Arg146->Lys	1.05	0.83
rCTXA1/T1	Truncated at Trp179	1.85	<0.01

[1]The absolute amount of each protein used in each ADP-ribosyltransferase assay (see Figure 4) was estimated by densitometric scanning of the stained SDS-polyacrylamide gel (Figure 4, panel A) containing identical amounts of each protein used in the assay. The autoradiogram of the gel containing the human erythrocyte membranes (Figure 4, Panel C) was subsequently scanned to determine the radiographic density of the G protein alpha subunit ribosylated by each CTXA1 protein preparation. The density of the G protein band resulting from ADP-ribosylation with commercial CTXA1 without added urea was taken as 1.00 and the density of the band resulting form ribosylation by the other CTXA1 proteins was related to this preparation as a percentage of its density. These fractions were then normalized to 1.00 µg of added CTXA1 protein based on the densitometric of the stained gel to obtain an approximate, relative specific activity.

[2]The amount of commercial CTXA1 (without added urea) in the assay was taken as 1.00 µg.

[3]The radiographic density of the G protein alpha subunit ADP-ribosylated by the commercial CTXA1 (without added urea) was taken as 1.00.

Figure 4 shows a stained SDS-polyacrylamide gel (Panel A) of inclusion-body preparations of rCTXA1 and its site-specific analogs. An amount of protein identical to that shown in this gel was used to catalyze the individual ADP-ribosyltransferase reactions. Trichloroacetic acid (TCA) precipitates from these reactions were also run in SDS-PAGE and the gels subjected to autoradiography to illuminate the [^{32}P]ADP-ribose-labeled substrates. Panel B illustrates the results of the reactions without added G protein-containing human erythrocyte membrane preparation and Panel C shows the reactions with this added substrate.

The most important finding of these experiments is found in Figure 4, Panel C (and confirmed in Panel B): certain site-specific amino acid residue substitutions result in diminishment and, in some cases, apparently complete loss of enzyme activity as measured in this assay. In this regard, rCTXA1/L7 (Arg^{9}→Lys), rCTXA1/1B (His^{44}→Asn) and rCTXA1/1D (Glu^{112}→Gln) analog subunits appear to possess virtually no enzyme activity, whereas analogs rCTXA1/1C (His^{70}→Asn) and rCTXA1/1F (Asp^{9}→Glu) appear to have reduced activity when compared with both native CTXA (with urea) and rCTX1/A7 (no mutation other than the methionine residue at the amino terminus). Truncation at Trp^{179} (rCTXA1/T1/1156) also results in an analog A subunit with severely diminished enzyme activity.

Although these autoradiographic assays of enzyme activity are not strictly quantitative, we have attempted to derive a quantitative assessment from the gel and autoradiograms of Figure 4 to illustrate in a numerical sense what can be visually observed. This evaluation is found in Table 2. Here, we subjected the stained SDS-polyacrylamide gel (Fig. 4, Panel A),

containing rCTXA1 and each of the analogs described previously, to integrative scanning densitometry to more accurately assess the relative amount of each protein added to the assay; these were related to the amount of A1 subunit in native CTXA (without urea) added to the assay, taken as a value of 1.00 µg. Although an attempt was made to add equivalent amounts of each protein to the assays (estimated on the basis of the percentage of subunit protein in each inclusion body preparation), it can be seen that this estimation may have lacked precision. The autoradiogram of the subsequent enzyme reactions with G protein substrate (Fig. 4, Panel C) was also subjected to densitometry to determine the relative density of the radiographic image of the radiolabeled G protein α subunit band with that labeled by native CTXA (no urea) taken as 100%. An approximate-relative specific activity was then calculated by dividing the image density by the amount of added enzyme, with the specific activity of native CTX (without urea) taken as 1.00. It should be noted that the results of this type of quantitation are subject to certain experimental limitations (e.g., assumption of equal dye staining by each of the subunit preparations, band selection and circumscription for digitized densitometry, densitometer response characteristics, and assumption of a linear relationship between [^{32}P]ADP-ribose labelling and radiographic density). Nevertheless, the results (Table 2) illustrate in a numerical manner what can be visually observed in the autoradiograms: marked diminishment of enzyme activity in analogs rCTXA1/1C ($His^{70}\rightarrow Asn$), rCTXA1/1F ($Asp^{9}\rightarrow Glu$), and rCTXA1/T1 (Trp^{179} truncation) and virtual loss of activity by analogs rCTXA1/L7 ($Arg^{9}\rightarrow Lsy$), rCTXA1/1B ($His^{44}\rightarrow Asn$), and rCTXA1/1D ($Glu^{112}\rightarrow Gln$).

In the case in which no exogenous substrate is added (Figure 4, Panel B), both native CTXA and the enzymatically-active CTXA1 proteins can be seen to be autocatalytic, i.e., to catalyze the hydrolysis of NAD and the transfer of ADP-ribose to the enzyme itself (either in *cis*, in *trans*, or both). Multiple bands seen in the autoradiogram may be due to contaminating *E. coli* proteins capable of being ADP-ribosylated; alternatively, yet unlikely, they may represent minor variants of the subunit proteins (e.g., proteolytically-nicked or, perhaps, variants possessing some residual secondary structure in SDS). Recombinant CTXA1 preparations appear much more capable of participating in the autocatalytic process than does the A subunit of native CTX. The reasons for this increased autoribosylation are not presently understood, although it may be related to lack of substrate specificity by the yet-to-be-renatured recombinant protein, exposure of a sensitive ribosylation site in the recombinant protein as a result of improper secondary structure (no attempt was made in this particular experiment to achieve native conformation), or to the presence of ARFs (ADP-ribosylation factors) (31-37) in the crude recombinant preparations that stabilize the autocatalysis. However, when G protein substrate is added in the form of human erythrocyte membranes (Panel C), the focus of the ADP-ribosyltransferase reaction is shifted to this substrate, quenching autoribosylation.

Figure 5 demonstrates that the same general pattern of diminishment and loss of enzyme activity seen with the rCTXA1 analogs is also observed when the same residue substitutions are made in rCTXA versions of the recombinant subunit (i.e., versions with the A2 "tail" still covalently linked). However, the presence of the A2 region appears to significantly reduce the

ADP-ribosyltransferase of the enzymatically-active proteins. This reduction is more clearly illustrated in Figure 6, in which identical amounts of rCTXA and rCTXA1 are evaluated in the enzyme assay (Panel A), the radiolabeled products run on the same gel, and consequently subjected to equivalent autoradiographic exposure times (Panel B). As can be seen, rCTXA1 appears to possess greater activity than rCTXA (compare lanes 7 and 4). Again, neither subunit construction with the $Arg^{9}\rightarrow Lys$ substitution (lanes 5 and 8) possess measurable ADP-ribosyltransferase activity for the G protein substrate. That this loss of enzyme activity in the analogs is not the result of *E. coli* contaminants suppressing catalysis is evident by the ability of native CTXA to ribosylate G protein in the presence of the *E. coli*-produced, analog-containing preparations (lanes 6 and 9).

Because of their reduction or essential elimination of a major marker of toxic activity (ADP-ribosyltransferase), the recombinant CTXA1 analog molecules produced by clones pCTXA1/L7/1156, pCTXA1/1B/1156, pCTXA1/1C/1156, pCTXA1/1D/1156, pCTXA1/1F/1156, and pCTXA1/T1/1156, as well as their rCTXA analog counterparts, are anticipated to have application alone or in combination with CTXB in safer vaccines. The described mutations would not be expected to reduce the normal, protective, immunogenic properties of native CTX subunits. The CTXA and CTXA1 analogs of this invention thus have application in combination with CTXB subunits in the form of a holotoxoid. The CTXB subunits may augment the immune response to CTXA and CTXA1, and vice-versa, and each may have protective epitopes. The CTXB subunits can be derived from *V. cholerae* or can be genetically-engineered subunits and their analogs. Genetically-

engineered subunit products can include fusion proteins and non-fusion proteins.

Strategies identical to those described above were employed to prepare additional recombinant analogs of the CTXA subunit of cholera toxin. The synthetic oligonucleotides utilized to effect codon substitutions, whether by linker mutagenesis or by mutagenesis by site-directed priming, are shown in Table 3. Briefly, analog CTXA1/1J ($Asp^{9}\rightarrow Tyr$) was prepared by linker mutagenesis as described for analog CTXA1/1F ($Asp^{9}\rightarrow Glu$), with the exception that the synthetic oligonucleotide possessed the appropriate codon substitution. For the construction of analogs CTXA1/1K ($Ser^{10}\rightarrow Gly$), CTXA1/1L ($Arg^{11}\rightarrow Lys$), and CTXA1/1M ($Arg^{11}\rightarrow His$), a novel DraII (also known as Eco0109I) restriction site was introduced into the CTXA1 gene by site-directed priming utilizing the following synthetic oligonucleotide primer:

5'-AGCAGTCAGGGGGCCTTATGCCAA-3'

Introduction of this site permitted linker mutagenesis in this region of the gene (using the previously-described NdeI site to the left of the insertion site and the newly-created DraII site to the right of the insertion site) to effect the codon changes that resulted in these three analogs. Site-directed priming was the method used to create the codon changes resulting in analogs CTXA1/1N ($His^{44}\rightarrow Tyr$), CTXA1/1"O" ($His^{44}\rightarrow Gln$), CTXA1/1P ($His^{44}\rightarrow Val$), CTXA1/1Q ($His^{70}\rightarrow Tyr$), CTXA1/1R ($His^{70}\rightarrow Gln$), and CTXA1/1S ($His^{70}\rightarrow Val$).

With two exceptions, each of these analogs was expressed in recombinant *E. coli* and the isolated inclusion bodies were tested for their enzymatic ability to ADP-ribosylate either $G_s\alpha$ in human

erythrocyte membrane preparations or, especially in the case of the His^{44} and His^{70} analogs, their ability to ADP-ribosylate $G_s\alpha$ and/or tubulin in membrane preparations of H27 cultured human foreskin fibroblasts (provided by the University of California, San Francisco). The exceptions were for analog CTXA1/1J ($Asp^{9}\rightarrow Tyr$), which was recombinantly expressed but not assayed for activity, and analog CTXA1/1L ($Arg^{11}\rightarrow Lys$), for which a linker had been synthesized and cloning performed, but for which a correct-sequence clone had not been isolated.

The results of these analyses are presented in Figures 4 and 7, and are summarized in Table 4, Figure 4 provides comparative data for analogs reported in Table 1. Among the additional analogs described in Figure 7 and Table 4 are three having different substitutions at His^{44} (CTXA1/1N, CTXA1/1"O", CTXA1/1P). The absence of measurable enzyme activity in these analogs, in addition to lack of activity in previously-described analog CTXA1/1B ($His^{44}\rightarrow Asn$), indicates that these specific substitutions at His^{44} lead to inactivation of the subunit of cholera toxin possessing the intrinsic toxic activity of the multimeric molecule. Based on these results, it is likely that *any* substitution at this residue will produce such inactivation.

Three analogs (CTXA1/1Q, CTXA1/1R, CTXA1/1S) having substitutions for His^{70} are also among those described. These analogs are in addition to the analog CTXA1/1C ($His^{70}\rightarrow Asn$) of Table 1. As shown in Figure 7, all four His^{70} analogs possess *reduced* ability to ADP-ribosylate $G_s\alpha$ substrate, although they clearly *retain* the ability to ADP-ribosylate other non-$G_s\alpha$ protein substrates (e.g., tubulin in H27 fibroblasts). Thus, substitutions for His^{70} result in apparent reduction of activity of CTXA1 for the

specific $G_s\alpha$ substrate believed to be involved in the pathognomonic cytotoxic response to cholera toxin. Such substitutions, if made in CTXA1 involved in a formed holotoxin multimer, would therefore likely result in an *attenuated* cholera toxin molecule as opposed to one totally lacking toxic properties.

Analysis of two additional analogs is shown in Figure 8. CTXA1/1K ($Ser^{10}\rightarrow Gly$) retains the catalytic activity associated with the native CTXA molecule. Substitution of His for Arg^{11} (CTXA1/1M) results in an analog having little or no measurable enzymatic activity. It would be expected that analog CTXA1/1L ($Arg^{11}\rightarrow Lys$) will also have significantly diminished activity when isolated and assayed, a conclusion which is supported by the findings of Table 1, (see $Arg^{7}\rightarrow Lys$).

TABLE 3
CONSTRUCTION OF CTXA1 ANALOGS

ANALOG	MUTATION	TECHNIQUE
CTXA1/1J	Asp9->Tyr	Linker Insertion
CTXA1/1K	Ser10->Gly	Linker Insertion
CTXA1/1L	Arg11->Lys	Linker Insertion
CTXA1/1M	Arg11->His	Linker Insertion
CTXA1/1N	His44->Tyr	Site-directed Priming
CTXA1/1"O"	His44->Gln	Site-directed Priming
CTXA1/1P	His44->Val	Site-directed Priming
CTXA1/1Q	His70->Tyr	Site-directed Priming
CTXA1/1R	His70->Gln	Site-directed Priming
CTXA1/1S	His70->Val	Site-directed Priming

ANALOG	OLIGONUCLEOTIDE SEQUENCE
CTXA1/1J	TAT GAA TGA TGA TAA GTT ATA TCG GGC ATA CT A CTT ACT ACT ATT CAA TAT AGC CCG TAT GAG ATC
CTXA1/1K	TAT GAA TGA TGA TAA GTT ATA TCG GGC AGA TGG CAG ACC TCC TGA TGA AAT AAA GCA GTC AGG G A CTT ACT ACT ATT CAA TAT AGC CCG TCT ACC GTC TGG AGG ACT ACT TTA TTT CGT CAG TCC CCC G
CTXA1/1L	TAT GAA TGA TGA TAA GTT ATA TCG GGC AGA TTC TAA GCC TCC TGA TGA AAT AAA GCA GTC AGG G A CTT ACT ACT ATT CAA TAT AGC CCG TCT AAG ATT CGG AGG ACT ACT TTA TTT CGT CAG TCC CCC G
CTXA1/1M	TAT GAA TGA TGA TAA GTT ATA TCG GGC AGA TTC TCA CCC TCC TGA TGA AAT AAA GCA GTC AGG G A CTT ACT ACT ATT CAA TAT AGC CCG TCT AAG AGT GGG AGG ACT ACT TTA TTT CGT CAG TCC CCC G
CTXA1/1N	CTT TAT GAT TAC GCA AGA GGA
CTXA1/1"O"	CTT TAT GAT CAG GCA AGA GGA
CTXA1/1P	CTT TAT GAT GTT GCA AGA GGA
CTXA1/1Q	AGA AGT GCC TAC TTA GTG GGT
CTXA1/1R	AGA AGT GCC CAG TTA GTG GGT
CTXA1/1S	AGA AGT GCC GTT TTA GTG GGT

TABLE 4

ADP-Ribosyltransferase Activities of CTXA1 Analogs

ANALOG	MUTATION	ADP-ribosyltransferase Activity [1]
CTXA1/1J	Asp9->Tyr	N.D.
CTXA1/1K	Ser10->Gly	[+]
CTXA1/1L	Arg11->Lys	N.D.
CTXA1/1M	Arg11->His	[-]
CTXA1/1N	His44->Tyr	[-]
CTXA1/1"O"	His44->Gln	[-]
CTXA1/1P	His44->Val	[-]
CTXA1/1Q	His70->Tyr	[±]
CTXA1/1R	His70->Gln	[±]
CTXA1/1S	His70->Val	[±]

[1] As visualized by SDS-PAGE and autoradiography: [+], full activity; [±], reduced activity; [-], no detectable activity; N.D., not determined.

IN VITRO ASSOCIATION OF rCTX SUBUNITS

A number of methods by which native cholera toxin can be dissociated and the individual subunits reassociated *in vitro* to reform the holotoxin molecules have been described in the literature(36,37). *In vitro* reassociation of the subunits of pertussis toxin has also been described in the literature for native subunits(38-40). Using a similar procedure, recombinant CTX subunits can be isolated, associated *in vitro* to form holotoxin-like species, and purified. In general, following expression and recovery, the individual subunits are combined in stoichiometric ratios (based on their relative content of specific subunit protein, if in the form of inclusion body preparations), approximating the ratio of subunits found in native CTX holotoxin. The preparation is solubilized in an aqueous solution containing a chaotropic agent or a detergent, or both. The preparation is subjected to reducing conditions (generally a reducing agent or a hydrogen atmosphere, or both) and then oxidized (with either an oxidizing agent or under an oxygen-enriched atmosphere, or both) to reform the necessary intramolecular disulfide bridges. Association of the subunits into holotoxin-like species is accomplished by diminishment or removal of the chaotropic or detergent solubilizing agent. This can be accomplished by a variety of means, to include filtration and buffer exchange by dialysis chromatography. The holotoxin-like species are then purified by conventional means, e.g., ion exchange, size-exclusion and affinity chromatography. It should be noted that B multimeric species, without the A subunit, may be recovered by similar means if inclusion-body preparations of the latter subunit are not added.

The genetically engineered analog subunits of this invention can be formulated, in a conventional manner, into a toxoided cholera vaccine. In the case of a toxin that has been "genetically" inactivated, such as cholera toxin in the present invention, further inactivating steps (such as chemical treatment or heat treatment) should not usually be required since these products are produced in non-pathogenic organisms and are inherently free of the enzyme activities that are generally accepted to elicit the adverse reactions to whole-cell cholera vaccines. Nevertheless, it is necessary to control purity of the recombinant product, particularly with regard to the endotoxin content. In general, recombinant holotoxoid, recombinant holotoxoid-like macromolecules, recombinant B subunit macromolecules, recombinant B subunit alone or possibly B subunit recombinant analogs, and even A subunit analogs alone described in the present invention as potential vaccinating antigens would be purified to ≥90% homogeneity. The nature and estimated quantity of contaminants, if any, would be evaluated to ensure that the extent of endotoxin contamination meets the standards of the individual regulatory agencies.

For purposes of parenteral delivery, the vaccine materials would normally be adsorbed onto aluminum adjuvants. This can be accomplished by at least two means: precipitation with preformed alum and precipitation with aluminum salts. The adsorbed precipitates are then resuspended in an excipient to yield a dosage concentration of vaccine antigen generally in the range of 5-100 µg per dose and an alum amount usually not exceeding 1.5 mg/dose; volume per dose is in the range of 0.1-1.0 ml. The suspending excipient is commonly a buffered solution (e.g., phosphate-buffered saline, pH 7.0), may have added stabilizers (e.g., glycerol), and will likely contain a

preservative (e.g., 0.01% Thimerosol) to prevent microbial contamination and to extend shelf life.

The formulation and delivery of recombinant cholera toxoid, or subcomponents thereof, *via* live vector systems as also encompassed within this invention will depend upon the nature of that system. For example, oral delivery of recombinant (mutant) *V. cholerae*, *Salmonella sp.*, vaccinia virus, or adenovirus carrying genes for the A or A *and* B subunits, might well be encapsulated in enteric-coated delivery vehicles for passage to the gut or in aerosolizable forms (e.g., with liposomes) for targeting to the respiratory tract in order to elicit secretory immunoglobulin A antibodies for protection at mucosal surfaces. Alternatively, other oral forms of the vaccine can be prepared in accordance with procedures described in the literature, suitably adapted to accommodate the present antigenic agents. For instance, a recombinant *V. cholerae* strain can be lyophilized and mixed with a bicarbonate buffer to neutralize gastric acidity(41); or a holotoxoid in accordance with this invention can be used in the form of an effervescent tablet, appropriately buffered, to supplement a killed, whole-cell vaccine(1).

* * *

While this invention has been specifically illustrated in relation to recombinant production in *E. coli*, it will be appreciated by those skilled in the art that the principles for mutagenesis of the analog subunits as described herein may be employed in connection with other recombinant hosts and expression systems, and to produce other inactivated analogs of the toxin. Further, it should be understood that assembly of mutant analogs into a holotoxoid can take place in intact cells via homologous recombination, e.g., in *V. cholerae*, rather than *in vitro*. It is intended that the present invention include all modifications and improvements as come within the scope of the present invention as claimed.

BIBLIOGRAPHY

1. Holmgren, J. et al. (1989) *Vaccine* **7**:94-96.

2. Long A.R., *Los Angeles Times*, April 21, 1991, A1-A5.

3. Levine, M.M. et al. (1983) *Microbiol. Rev.*, **47**:510-550.

4. Pierce, N.F. et al. (1985) *Infect. Immun.* ***50***:813-816.

5. Finkelstein, R.A. (1988) in *Immunochemical and molecular genetics of Bacterial pathogens* (Owen P. and Foster T.J., eds.), pp. 85-102, Elsevier Science Publishers, North Holland, The Netherlands.

6. Fishman, P.H. (1990) in *ADP-Ribosylating Toxins and G Proteins*, (Moss J. and Vaughan M. eds.) pp. 127-140, *American Society for Microbiology*, Washington D.C.

7. United States Patent No. 4,666,837 (Harford et al.).

8. United States Patent No. 4,328,209 (Honda et al.)

9. Kaper, J.B. et al. (1984) *Nature* ***308***:655-658.

10. United States Patent No. 4,751,064 (Sela et al.).

11. Black, R.E. et al. (1987) *Infect. Immun.* ***55***:1116-1120.

12. Spriggs, D.R., and Sack, R.B. (1990) *J. Inf. Dis.* ***162***:584-590.

13. Kaper, J.B., and Levine, M.M. (1990) *Res. Microbiol.* ***141***:901-906.

14. Duffey, L.K. et al. (1981) *FEBS Lett.* ***126***:187-190.

15. Mekalanos, J.J. et al. (1979) *J. Biol. Chem.* ***254***:5855-5861.

16. Mekalanos, J.J. (1983) *Nature* ***306***:551-557.

17. Yamamoto, T. et al. (1984) *FEBS Lett.* ***169***:241-246.

18. Matteuci, M.D., and Caruthers, M.H. (1981) *J. Am. Chem. Soc.* ***103***:3185ff.

19. Bachmann, B.J. et al. (1976) *Bacteriol. Rev.* ***40***:116-167.

20. Sussman, R., and Jacob, F. (1962) *C.R. Acad. Sci;* ***254***:1517-1579.

21. Sambrook, J. et al. (1989) *Molecular cloning: a laboratory manual,* 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

22. Sanger, F. et al. (1977) *Proc Natl. Acad. Sci., USA* ***74***:5463-5467.

23. Heidecker, G. et al. (1980) *Gene* ***10***:69-73.

24. Hewick, R.M. et al. (1981) *J. Biol. Chem.* ***256***:7990-7997.

25. Hunkapillar, M.W. et al. (1983) *Meth. Enzymol.* ***91***:399-413.

26. Laemmli, U.K. (1970) *Nature* ***227***:680-685.

27. Hunkapillar, M.W. et al., *supra*, at pp. 227-236.

28. Kaslow, H.R. et al. (1980) *J. Biol. Chem.* ***255***:3786-3741.

29. Fling, S.P., and Gregerson, D.S. (1986) *Anal. Biochem.* ***155***:83-88.

30. Burnette, W.N. et al. (1988) *Science* ***242***:72-74.

31. Kahn, R.A., and Gilman, A.G. (1984) *J. Biol. Chem.* ***259***:6228-6234.

32. Kahn, R.A. and Gilman, A.G. (1986) *J. Biol. Chem.* ***261***:7906-7911.

33. Tsai, S.-C. et al. (1987) *Proc. Natl. Acad. Sci., USA* ***84***:5139-5142.

34. Tsai, S.-C. et al. (1988) *J. Biol. Chem.* ***263***:1768-1772.

35. Bovak, D.A. et al. (1990) *Biochemistry* ***29***:855-861.

36. Hardy, S.J.S. et al. (1988) *Proc. Natl. Sci., USA* ***85***:7109-7113.

37. Finkelstein, R.A. et al. (1974) *J. Immunol.* ***113***:145-150.

38. Tamura, M. et al. (1982) *Biochem.* ***21***:5516-5522.

39. Bartley, T.D. et al. (1989) *Proc. Natl. Acad. Sci., USA* ***86***:8353-8357.

40. Yamakawa, Y. et al. (1990) *Anal. Biochem.* ***185***:176-181.

41. Cryz, S. J. et al. (1990) *Vaccine* ***8***:577-580.

WHAT IS CLAIMED:

1. A recombinant DNA molecule, at least a portion of which encodes an analog of the catalytic subunit of cholera toxin, wherein said analog has reduced or no catalytic activity associated with cholera toxin reactogenicity.

2. The recombinant DNA molecule of claim 1, wherein the analog is of the A region of cholera toxin.

3. The recombinant DNA molecule of claim 1, wherein the analog is of the A1 subunit of cholera toxin.

4. The recombinant DNA molecule of claim 1, wherein the analog is capable of eliciting a cholera toxin-neutralizing immune response.

5. The recombinant DNA molecule of claim 1, which is obtained by site-specific mutagenesis resulting in an analog of the catalytic subunit which is less active or essentially inactive as determined by assay of ADP-ribosyltransferase activity.

6. The recombinant DNA molecule of claim 5, wherein the site-specific mutation is in the region bounded by the codons for methionine-1 and arginine-192 or serine-194, inclusively.

7. The recombinant DNA molecule of claim 6, which encodes an analog of the catalytic subunit comprising a site-specific mutation in one or more of the sites of said subunit selected from among arginine-7, arginine-11, aspartic acid-9, histidine-44, histidine-70 and glutamic acid-112.

8. The recombinant DNA molecule of claim 6, which encodes an analog of the catalytic subunit comprising a truncation of the carboxyl-terminal portion beginning at tryptophan-179.

9. The recombinant DNA molecule of claim 1, which also encodes subunit B of cholera toxin.

10. A genetically engineered analog of the catalytic subunit of cholera toxin, said analog having reduced or essentially no catalytic activity associated with cholera toxin reactogenicity.

11. The analog of claim 10, which is of the A region of cholera toxin.

12. The analog of claim 10, which is of the A1 subunit of cholera toxin.

13. The analog of claim 10, which is capable of eliciting a cholera toxin-neutralizing immune response.

14. The analog of claim 10, which is obtained by site-specific mutagenesis resulting in a mutation of the catalytic subunit which is less active or essentially inactive as determined by assay of ADP-ribosyltransferase activity.

15. The analog of claim 14, wherein the site-specific mutation is in the region bounded by methionine-1 and arginine-192 or serine-194, inclusively.

16. The analog of claim 15, which comprises a site-specific mutation in one or more of the sites of said subunit selected from among arginine-7, arginine-11, aspartic acid-9, histidine-44, histidine-70 and glutamic acid-112.

17. The analog of claim 15, which comprises a truncation of the carboxyl-terminal portion beginning at tryptophan-179.

18. An improved anti-cholera vaccine comprising an effective amount of an analog of the catalytic subunit of cholera toxin, wherein said toxin has a biological activity which (a) can elicit a cholera toxin-neutralizing immune response and (b) has reduced or essentially no catalytic activity associated with cholera toxin reactogenicity.

19. The improved vaccine of claim 18, wherein the analog is of the A region of cholera toxin.

20. The improved vaccine of claim 18, wherein the analog is of the A1 subunit of cholera toxin.

21. The improved vaccine of claim 18, wherein the toxin-neutralizing immune response provides immunoprotection against cholera disease.

22. The improved vaccine of claim 18, wherein the analog has been derived by site-specific mutagenesis resulting in a mutation of the catalytic subunit of cholera toxin which has less or essentially no ADP-ribosyltransferase activity.

23. The improved vaccine of claim 22, wherein the site-specific mutation is in the region of the catalytic subunit bounded by methionine-1 and arginine-192 or serine-194, inclusively.

24. The improved vaccine of claim 23, wherein the site-specific mutation is in one or more of the sites of said subunit selected from among arginine-7, arginine-11, aspartic acid-9, histidine-44, histidine-70, and glutamic acid-112.

25. The improved vaccine of claim 23, wherein the mutation comprises truncation of the carboxyl-terminal portion beginning at tryptophan-179.

26. The improved vaccine of claim 18, wherein the analog of the catalytic subunit is associated with the B oligomer.

27. The improved vaccine of claim 26, wherein the B oligomer is the native form.

28. The improved vaccine of claim 26, wherein the B oligomer has been genetically engineered.

29. A prokaryotic or eukaryotic cell transformed with a DNA molecule according to claim 1 which is capable of expressing the polypeptide product or products encoded by said DNA molecule.

30. An *E. coli* host cell according to claim 29.

31. A strain of *Vibrio cholerae* containing a genetically engineered toxin operon in which one or more specific amino acid residues responsible for cholera toxin reactogenicity have been altered or deleted by mutagenesis.

32. The strain of *Vibrio cholerae* according to claim 31, wherein the operon has been mutagenized to alter one or more amino acids selected from among arginine-7, arginine-11, aspartic acid-9, histidine-44, histidine-70, and glutamic acid-112, or to truncate the carboxyl terminal portion beginning at tryptophan-179.

33. A method for the production of an analog of the catalytic subunit of cholera toxin which has reduced or essentially no catalytic activity associated with cholera toxin reactogenicity, comprising:

(a) identifying one or more amino acid residues of the toxin which are associated with such catalytic activity;

(b) effecting site-directed mutagenesis of the toxin cistron or operon to remove or replace such residue or residues and produce a mutagenized cistron or operon, and

(c) expressing the mutagenized cistron or operon in a transformed organism to produce a toxoid characterized by reduced or essentially no catalytic activity.

34. An isolated, genetically engineered protein having substantially the amino acid sequence of Figure 1A starting with asparagine-1 of the mature sequence and ending with leucine-240.

35. The protein of claim 34 which has been derived by expression in a host other than *Vibrio cholerae*.

36. The protein of claim 35 which has been expressed in *E. coli*.

S
S
p
I
N
d
e
I
preA
mature A
1 ATGGTAAAGATAATATTTGTGTTTTTATTTCTTATCATCATTTTCATATGCAAATGAT
TACCATTTCTATTATAAACACAAAAAATAAAAGAATAGTAGTAAAAGTATACGTTTACTA 60
M V K I I F V F F I F L S S S F S Y Y A N D -
X
b
a
I
61 GATAAGTTATATCGGGCAGATTCTAGACCTCCTGATGAAATAAAGCAGTCAGGTGGTCTT
CTATTCAATATAGCCCGTCTAAGATCTGGAGGACTACTTTATTTCGTCAGTCCACCAGAA 120
D K L Y R A D S R P P D E I K Q S G G L -
T
S a B
c q c
a I 1
I I I
121 ATGCCAAGAGGACAGAGTGAGTACTTTGACCGAGGTACTCAAATGAATATCAACCTTTAT
TACGGTTCTCCTGTCTCACTCATGAAACTGGCTCCATGAGTTTACTTATAGTTGGAAATA 180
M P R G Q S E Y F D R G T Q M N I N L Y -
181 GATCATGCAAGAGGAACTCAGACGGGATTTGTTAGGCACGATGATGGATATGTTTCCACC
CTAGTACGTTCTCCTTGAGTCTGCCCTAAACAATCCGTGCTACTACCTATACAAAGGTGG 240
D H A R G T Q T G F V R H D D G Y V S T -

FIG. 1A

```
                                       Bsp1286I
                                       |    DraIII/PflMI
241 TCAATTAGTTTGAGAAGTGCCCACTTAGTGGGTCAAACTATATTGTCTGGTCATTCTACT 300
    AGTTAATCAAACTCTTCACGGGTGAATCACCCAGTTTGATATAACAGACCAGTAAGATGA
    S  I  S  L  R  S  A  H  L  V  G  Q  T  I  L  S  G  H  S  T  -

                                                    AflIII
                                                    |    NspHI
                                                    |    |    TaqI
301 TATTATATATATGTTATAGCCACTGCACCCAACATGTTTAACGTTAATGATGTATTAGGG 360
    ATAATATATATACAATATCGGTGACGTGGGTTGTACAAATTGCAATTACTACATAATCCC
    Y  Y  I  Y  V  I  A  T  A  P  N  M  F  N  V  N  D  V  L  G  -

361 GCATACAGTCCTCATCCAGATGAACAAGAAGTTTCTGCTTTAGGTGGGATTCCATACTCC 420
    CGTATGTCAGGAGTAGGTCTACTTGTTCTTCAAAGACGAAATCCACCCTAAGGTATGAGG
    A  Y  S  P  H  P  D  E  Q  E  V  S  A  L  G  G  I  P  Y  S  -
```

FIG. 1A CONT.'

```
         BstXI                Tth111I
421 CAAATATATGGATGGTATCGAGTTCATTTTGGGGTGCTTGATGAACAATTACATCGTAAT 480
    GTTTATATACCTACCATAGCTCAAGTAAAACCCCACGAACTACTTGTTAATGTAGCATTA
    Q  I  Y  G  W  Y  R  V  H  F  G  V  L  D  E  Q  L  H  R  N  -

                              GsuI                            BspMI
481 AGGGGCTACAGAGATAGATATTACAGTAACTTAGATATTGCTCCAGCAGCAGATGGTTAT 540
    TCCCCGATGTCTCTATCTATAATGTCATTGAATCTATAACGAGGTCGTCGTCTACCAATA
    R  G  Y  R  D  R  Y  Y  S  N  L  D  I  A  P  A  A  D  G  Y  -

                      BspMII           Ksp632I       DsaI
541 GGATTGGCAGGTTTCCCTCCGGAGCATAGAGCTTGGAGGGAAGAGCCGTGGATTCATCAT 600
    CCTAACCGTCCAAAGGGAGGCCTCGTATCTCGAACCTCCCTTCTCGGCACCTAAGTAGTA
    G  L  A  G  F  P  P  E  H  R  A  W  R  E  E  P  W  I  H  H  -
```

FIG. 1A CONT.'

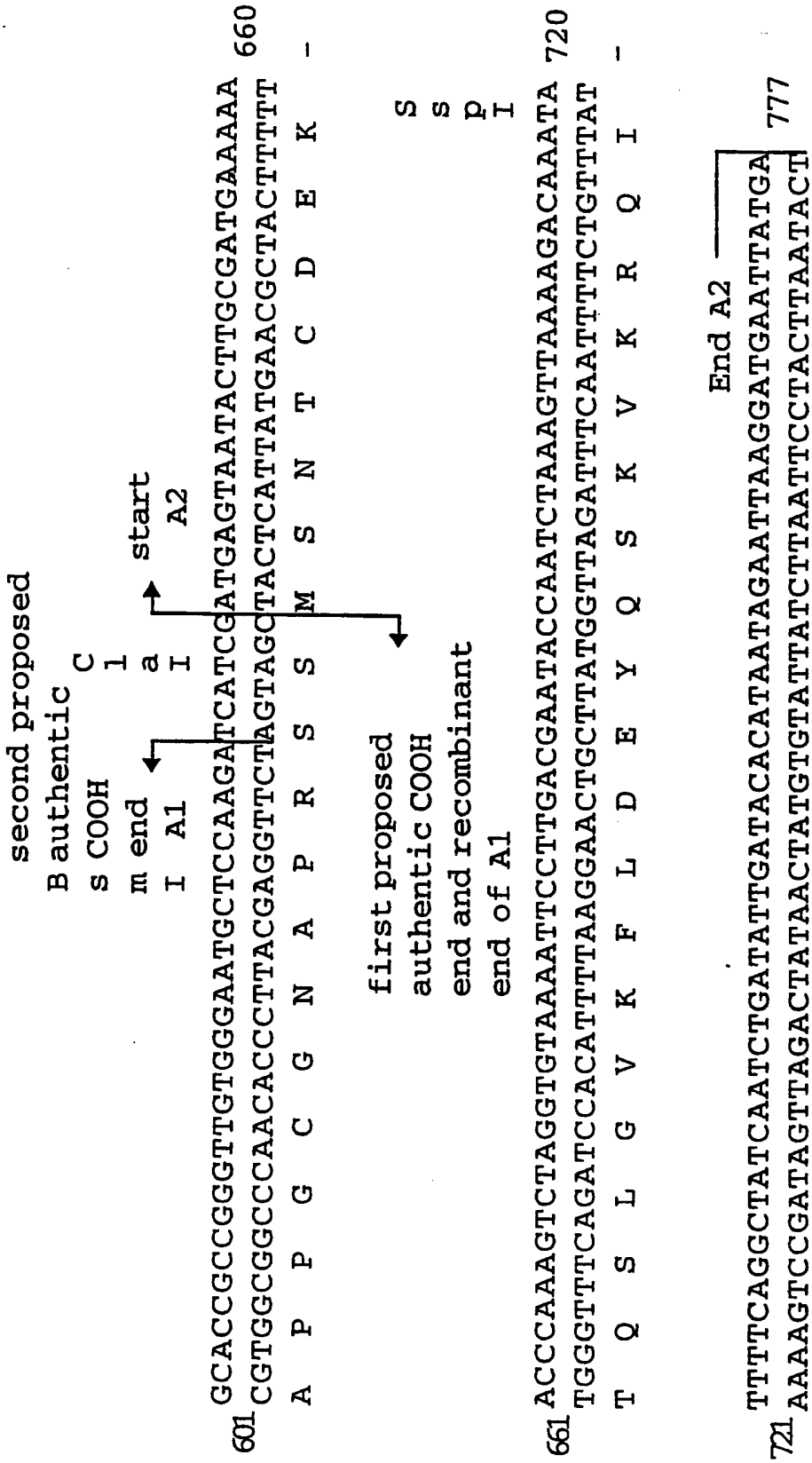

second proposed
B authentic
s COOH
m end
I A1
C
1
a
I
start
A2
first proposed
authentic COOH
end and recombinant
end of A1
S
s
p
I
End A2
601 GCACCGCCCGGGTTGTGGGAATGCTCCAAGATCATCGATGAGTAATACTTGCGATGAAAAA 660
CGTGGCGGGCCCAACACCCTTACGAGGTTCTAGTAGCTACTCATTATGAACGCTACTTTTT
A P P G G C G N A P R S S S M S N T C D E K -
661 ACCCAAAGTCTAGGTGTAAAATTCCTTGACGAATACCAATCTAAAGTTAAAAGACAAATA 720
TGGGTTTCAGATCCACATTTTAAGGAACTGCTTATGGTTAGATTTCAATTTTCTGTTTAT
T Q S L G V K F L D E Y Q S K V K R Q I -
721 TTTTCAGGCTATCAATCTGATATTGATACACATAATAGAATTAAGGATGAATTATGA 777
AAAAGTCCGATAGTTAGACTATAACTATGTGTATTATCTTAATTCCTACTTAATACT
F S G Y Q S D I D T H N R I K D E L * -

FIG. 1A CONT.'

```
                                     E
                                     c
                                     o
                                     5
     ┌──► PreB                       7
     │                               I
   1 ATGATTAAATTAAAATTTGGTGTTTTTTTTACAGTTTTACTATCTTCAGCATATGCACAT
     TACTAATTTAATTTTAAACCACAAAAAAAATGTCAAAATGATAGAAGTCGTATACGTGTA 60
     M  I  K  L  K  F  G  V  F  F  T  V  L  L  S  S  A  Y  A  T  -

                         S
         ┌──► Mature     s
         │       B       p
         │               I
  61 GGAACACCTCAAAATATTACTGATTTGTGTGCAGAATACCACAACACACAAATATATACG
     CCTTGTGGAGTTTTATAATGACTAAACACACGTCTTATGGTGTTGTGTGTTTATATATGC 120
     G  T  P  Q  N  I  T  D  L  C  A  E  Y  H  N  T  Q  I  Y  T  -

                                     AX
                                     cc
                                     ca
                                     II
 121 CTAAATGATAAGATATTTTCGTATACAGAATCTCTAGCTGGAAAAAGAGAGATGGCTATC
     GATTTACTATTCTATAAAAGCATATGTCTTAGAGATCGACCTTTTTCTCTCTACCGATAG 180
     L  N  D  K  I  F  S  Y  T  E  S  L  A  G  K  R  E  M  A  I  -
```

FIG. 1B

HincII

181 ATTACTTTTAAGAATGGTGCAATTTTTCAAGTAGAAGTACCAAGTAGTCAACATATAGAT 240
TAATGAAAATTCTTACCACGTTAAAAAGTTCATCTTCATGGTTCATCAGTTGTATATCTA
I T F K N G A I F Q V E V P S S Q H I D. -

AocI

241 TCACAAAAAAAAGCGATTGAAAGGATGAAGGATACCCTGAGGATTGCATATCTTACTGAA 300
AGTGTTTTTTTTCGCTAACTTTCCTACTTCCTATGGGACTCCTAACGTATAGAATGACTT
S Q K K A I E R M K D T L R I A Y L T E -

Eco57I

301 GCTAAAGTCGAAAAGTTATGTGTATGGAATAATAAAACGCCTCATGCGATTGCCGCAATT 360
CGATTTCAGCTTTTCAATACACATACCTTATTATTTTGCGGAGTACGCTAACGGCGTTAA
A K V E K L C V W N N K T P H A I A A I -

End B

361 AGTATGGCAAATTAA 375 375
TCATACCGTTTAATT
S M A N * -

FIG. 1B CONT.'

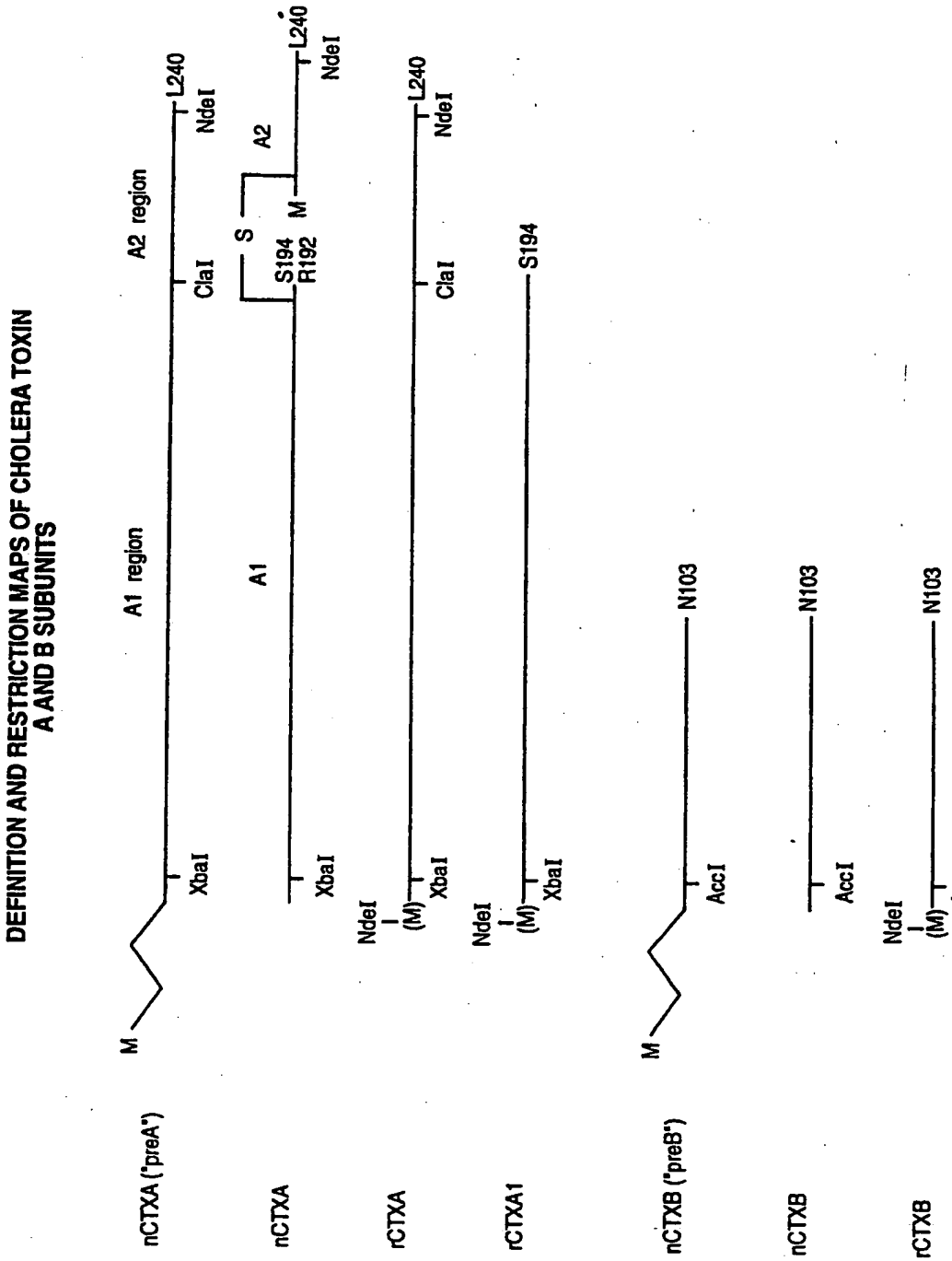

DEFINITION AND RESTRICTION MAPS OF CHOLERA TOXIN A AND B SUBUNITS
A1 region
A2 region
M
XbaI
ClaI
L240
NdeI
S
S194
R192
A2
A1
(M)
N103
AccI
nCTXA ("preA")
nCTXA
rCTXA
rCTXA1
nCTXB ("preB")
nCTXB
rCTXB

FIG. 2

FIG. 3

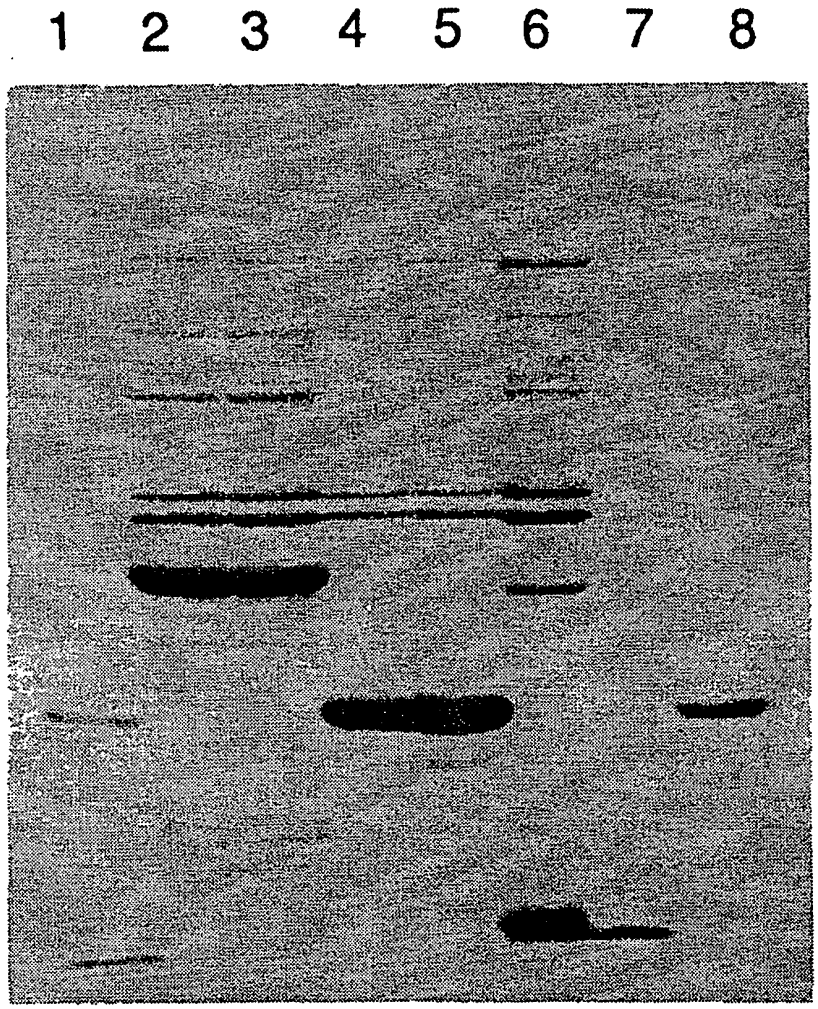


FIG. 4A

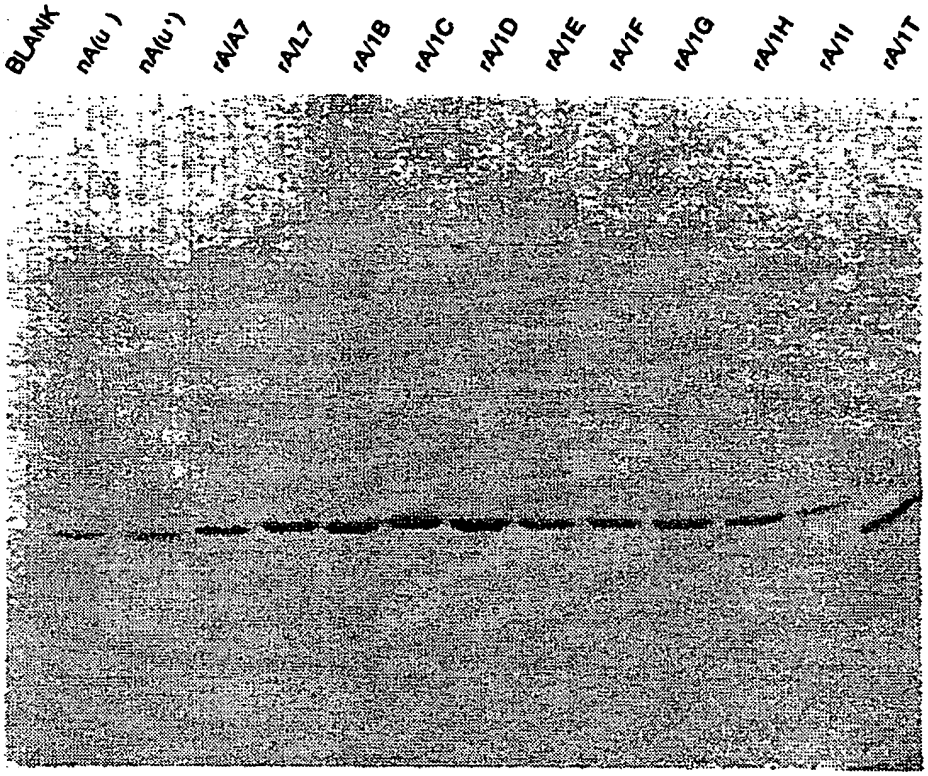

BLANK
rA(u)
rA(u')
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1G
rA/1H
rA/1I
rA/1T

FIG. 4B

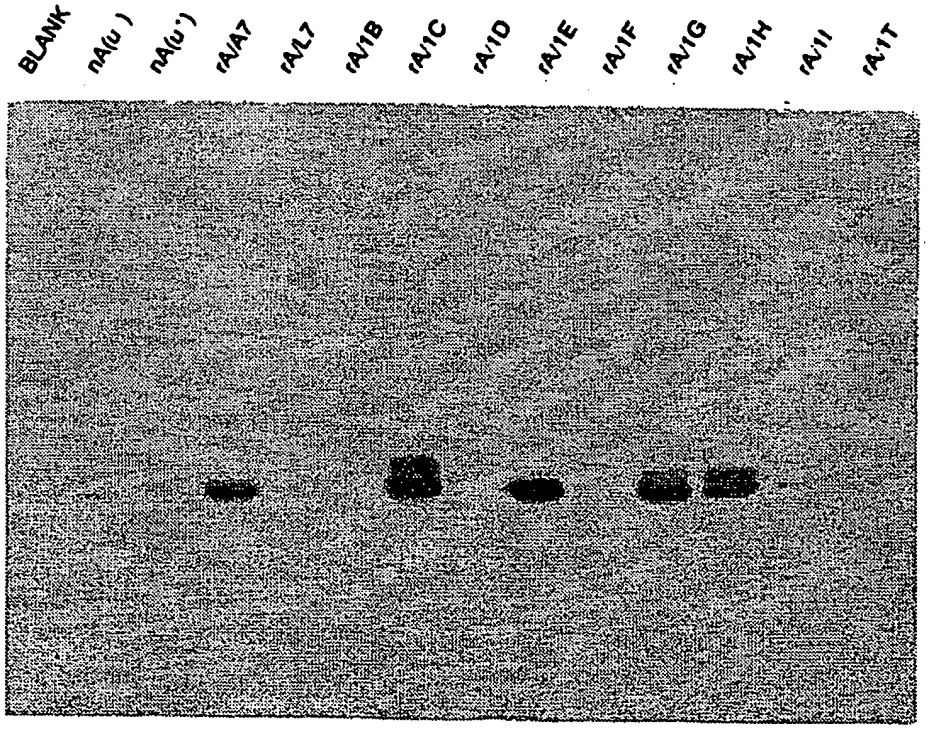

BLANK
rA(u)
rA(u')
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1G
rA/1H
rA/1I
rA/1T

FIG. 4C

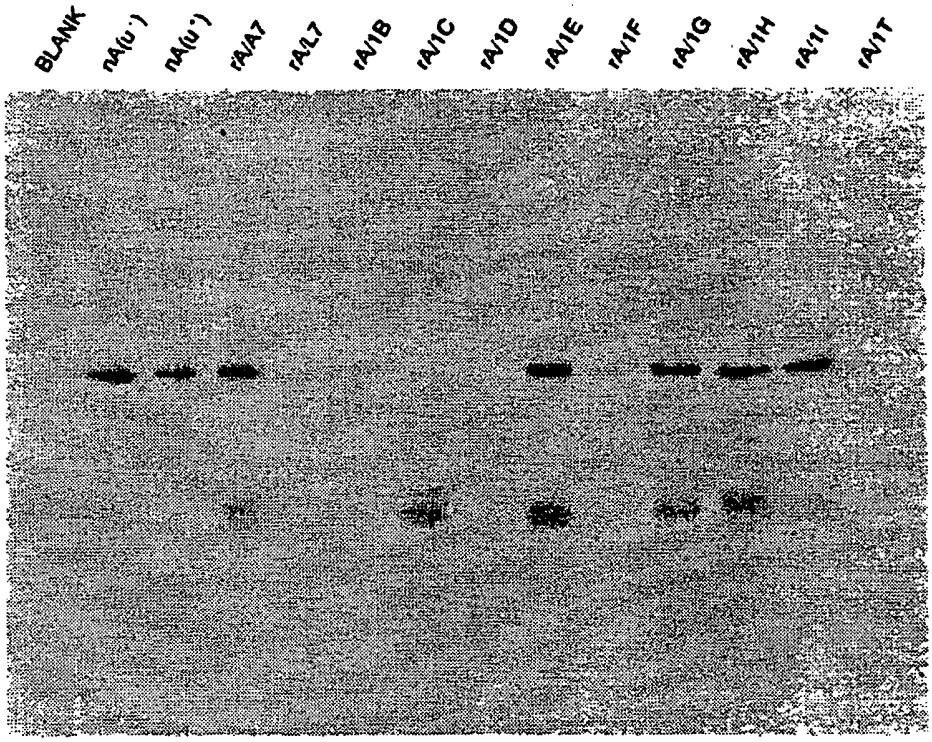

BLANK
nA(u⁻)
nA(u⁻)
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1G
rA/1H
rA/1I
rA/1T

FIG. 5A

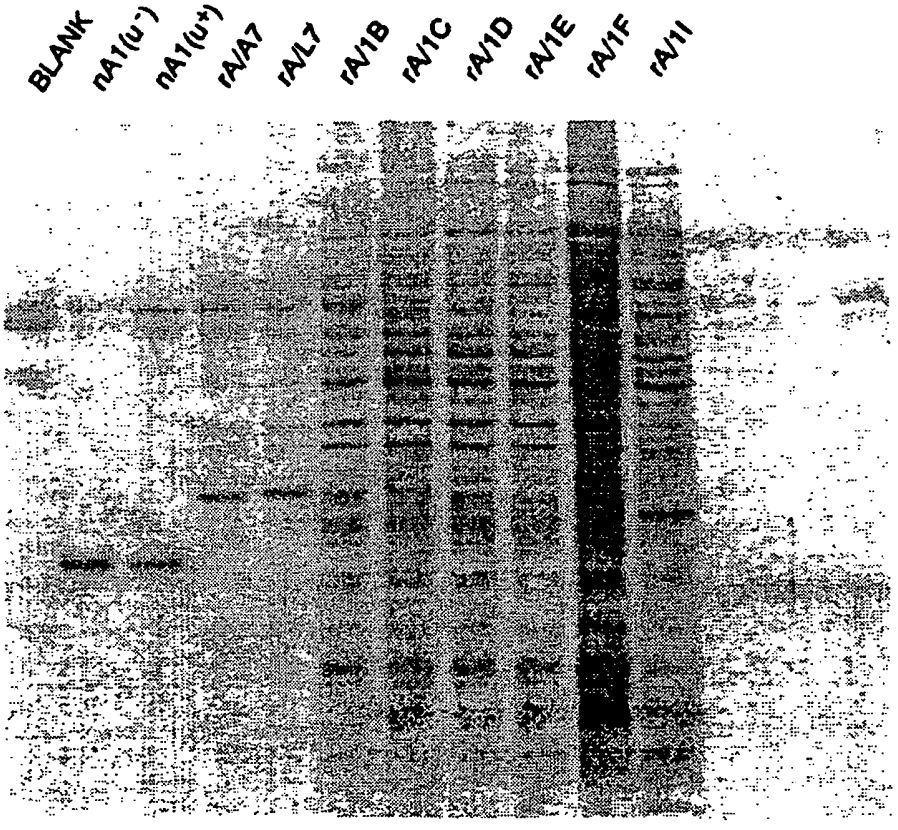

BLANK
nA1(u⁻)
nA1(u+)
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1I

FIG. 5B

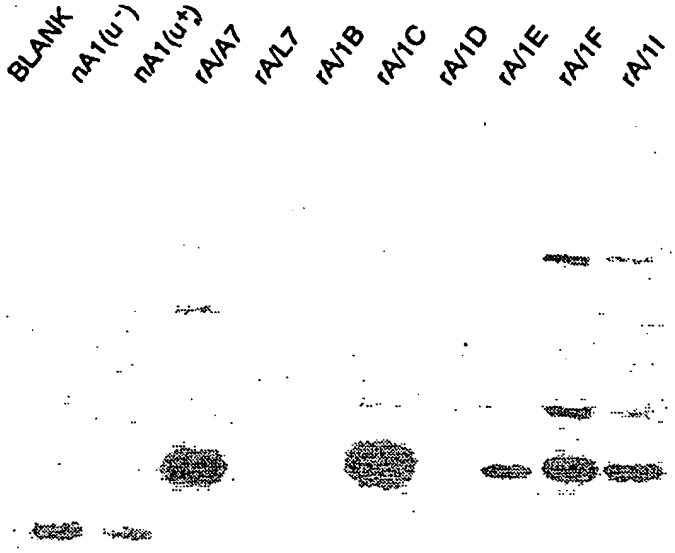

BLANK
nA1(u⁻)
nA1(u⁺)
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1I

FIG. 5C

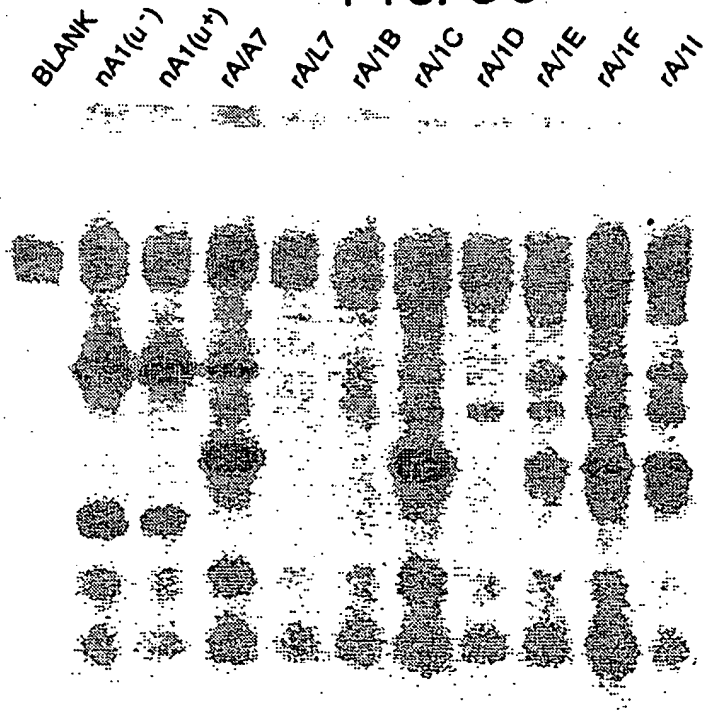

BLANK
nA1(u⁻)
nA1(u⁺)
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1I

FIG. 6A

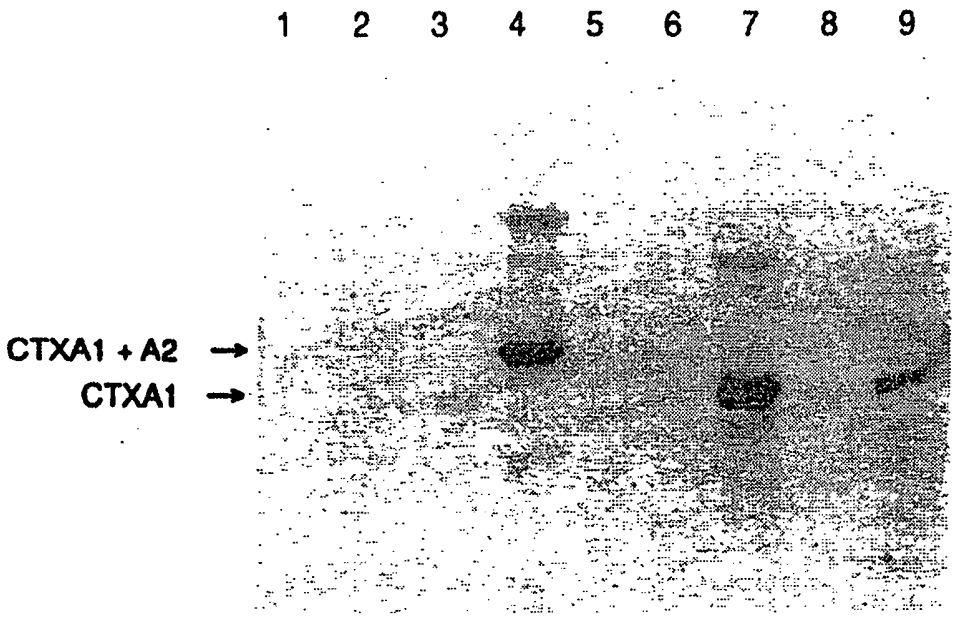
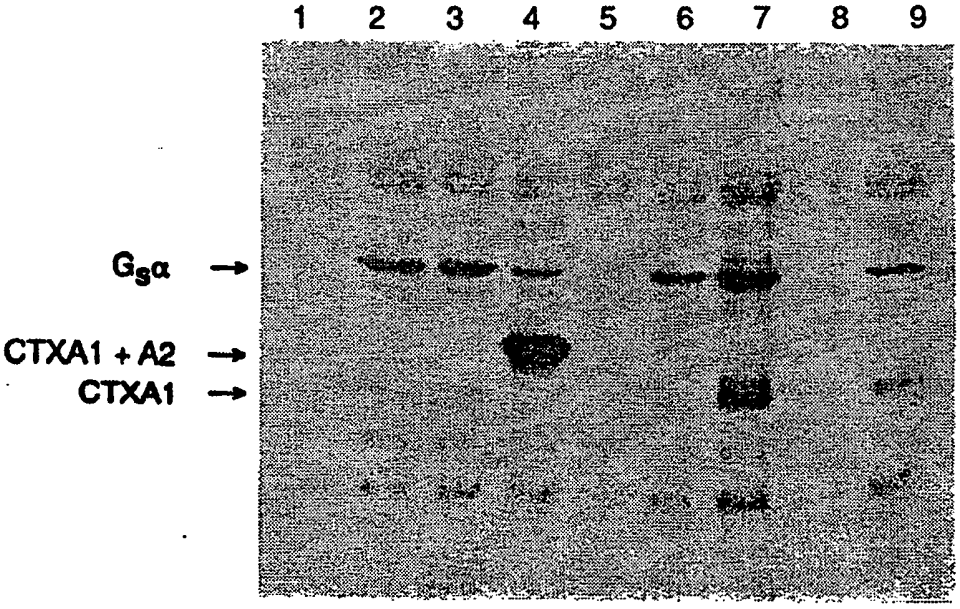


FIG. 6B



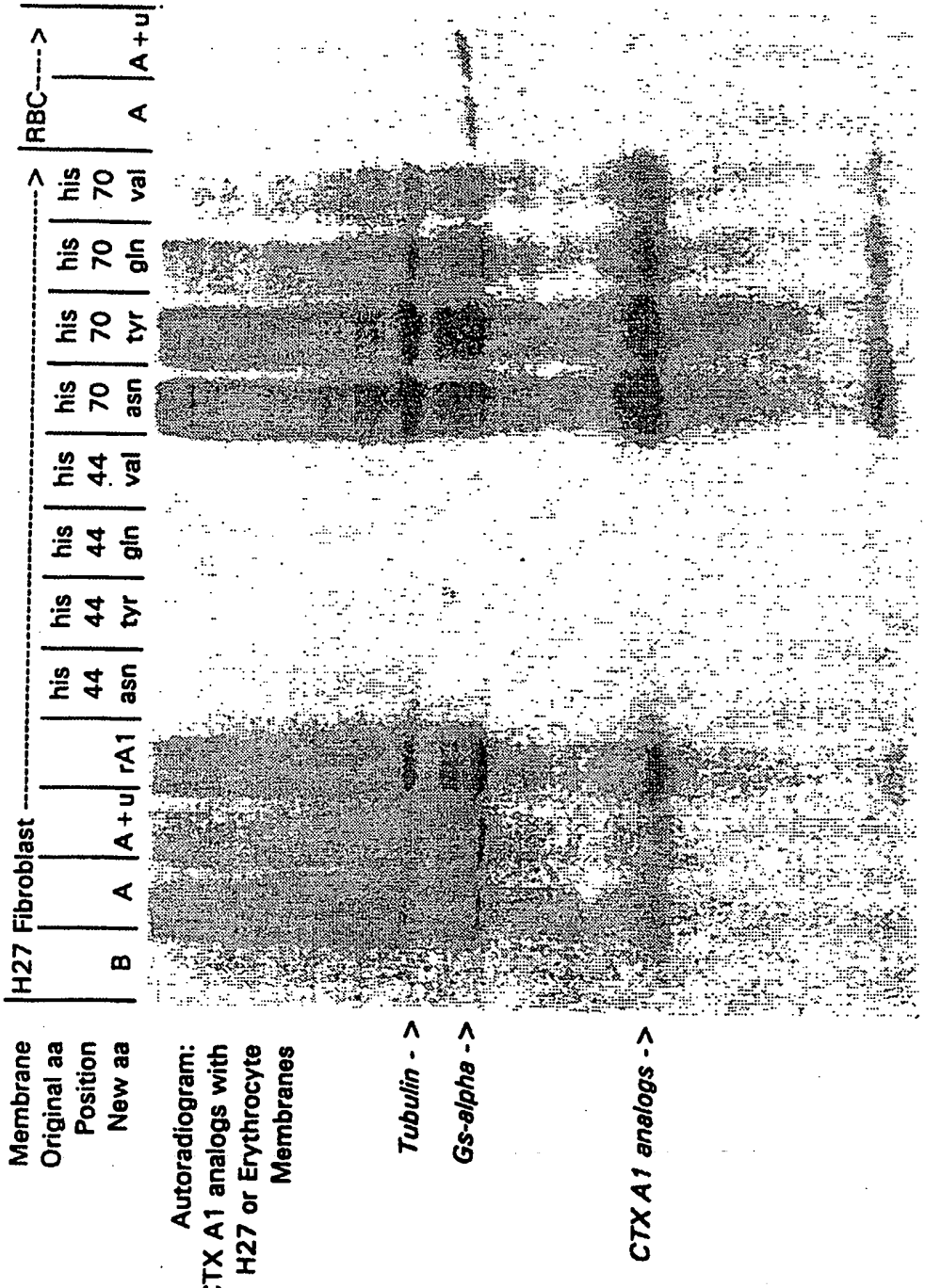

Membrane
Original aa
Position
New aa
H27 Fibroblast
B
A
A+u
rA1
his 44 asn
his 44 tyr
his 44 gln
his 44 val
his 70 asn
his 70 tyr
his 70 gln
his 70 val
RBC
A
A+u
Autoradiogram:
CTX A1 analogs with
H27 or Erythrocyte
Membranes
Tubulin ->
Gs-alpha ->
CTX A1 analogs ->

FIG. 7A


Membrane
Original aa
Position
New aa
H27 Fibroblast
B
A
A+u
rA1
arg 7 lys
his 44 asn
his 70 asn
glu 112 gln
tyr 6 phe
asp 9 glu
pro 185 gln
cys 187 ala
arg 146 lys
RBC
A
Autoradiogram:
CTX A1 analogs with
H27 or Erythrocyte
Membranes
Tubulin ->
Gs-alpha ->
CTX A1 analogs ->

FIG. 7B

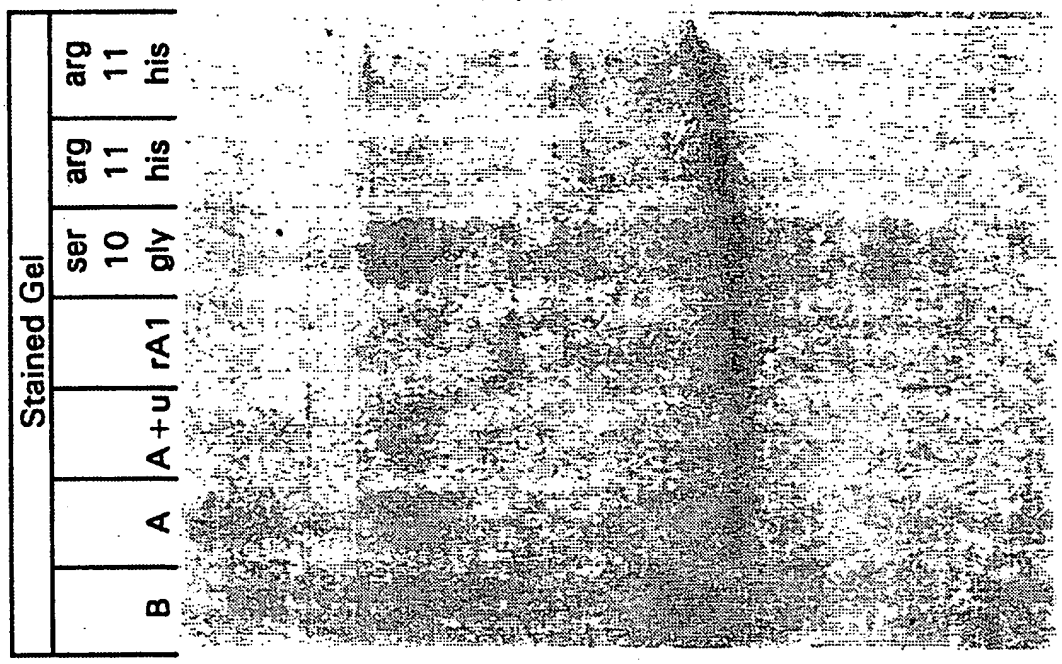

Stained Gel
B
A
A+u
rA1
ser 10 gly
arg 11 his
arg 11 his
Original aa
Position
New aa
CTX A1 analogs ->

FIG. 8A

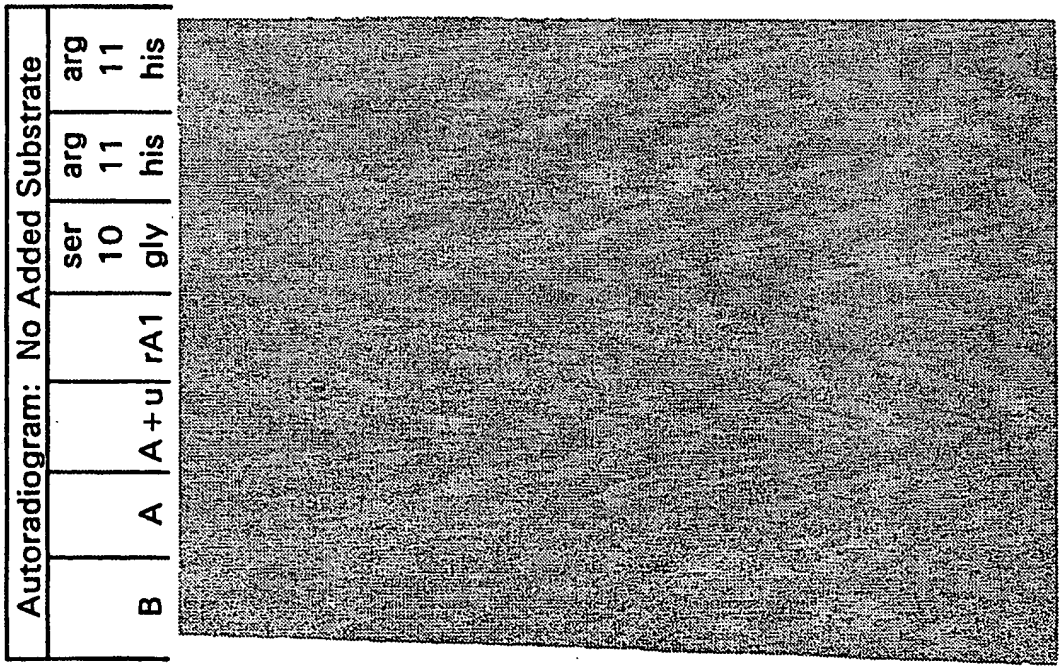

Autoradiogram: No Added Substrate
B
A
A+u
rA1
ser 10 gly
arg 11 his
arg 11 his

FIG. 8B

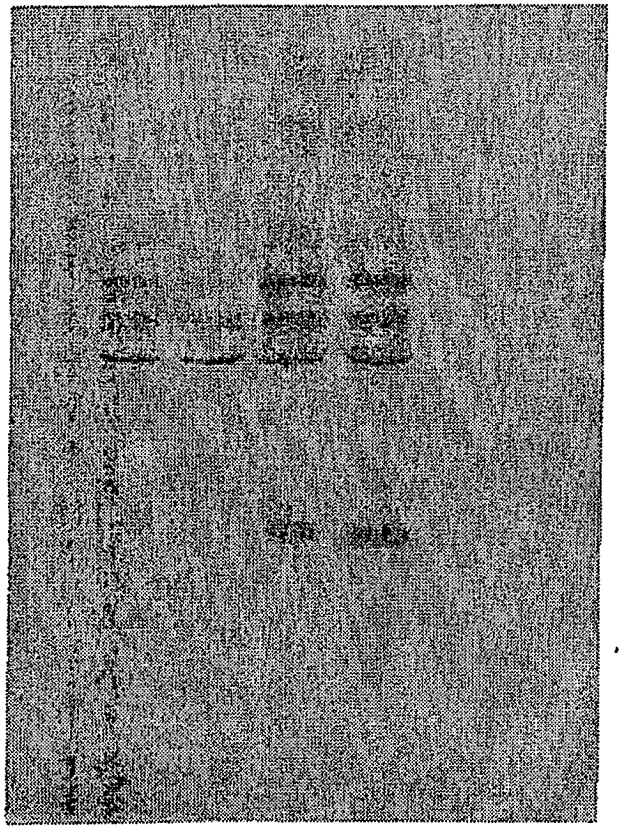


FIG. 8D

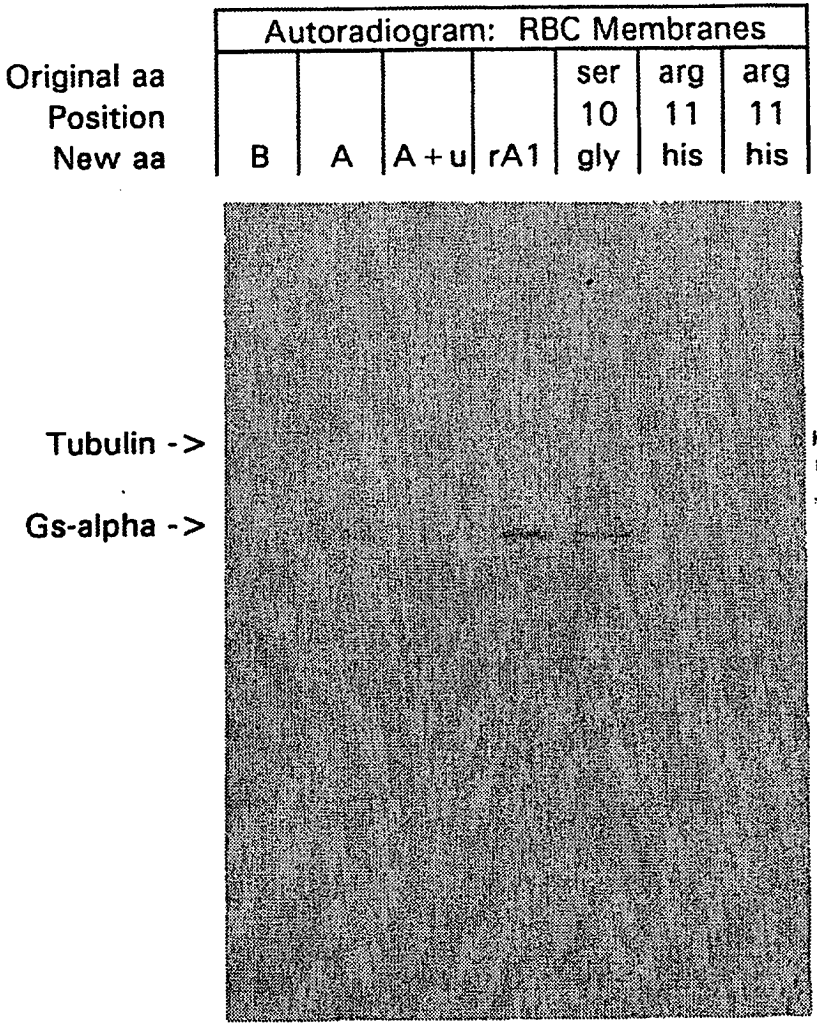


FIG. 8C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03703

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/106; C12N 1/20, 1/21, 5/10, 9/12, 15/31, 15/09; C12P 21/02
US CL :435/172.3, 194, 240.2, 252.1, 252.3, 252.33; 424/88; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 194, 240.2, 252.1, 252.3, 252.33, 909, 69.3, 71.1, 172.1; 424/88; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS
search terms: cholera toxin, reactogenicity, mutant, analog, toxoid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RESEARCH IN MICROBIOLOGY, Volume 141, issued 1990, J.B. Kaper et al., "Recombinant Attenuated Vibrio cholerae Strains Used as Live Oral Vaccines". pages 901-906, entire document especially pages 903-904.	1-6, 9-11, 13-15, 29, 31, 33
P,Y	CHEMICAL ABSTRACTS, Volume 115, No. 17, issued 28 October 1991, Y. Lobet et al., "Effect of Site-directed Mutagenic Alterations on ADP-Ribosyltransferase Activity of the A-subunit of Escherichia coli Heat-labile Enterotoxin", pages 433-434, abstract no. 178165, Infection and Immunity, 59(9), pages 2870-2879.	1-7, 9-16, 18-24, 26-33
X A	US, A, 4,935,364 (Kaper et al.) 19 June 1990, entire document.	33 1-32
X A	US, A, 4,882,278 (Mekalanos) 21 November 1989, entire document.	33 1-32
X Y	US, A, 4,666,837 (Harford et al.) 19 May 1987, entire document.	34-36 1-33
A	US, A, 4,328,209 (Finkelstein et al.) 04 May 1982.	18-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 August 1992	Date of mailing of the international search report 19 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer REBECCA PROUTY Telephone No. (703) 303-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03703

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X Y	CHEMICAL ABSTRACTS, Volume 116, No. 7, issued 17 February 1992, W. Burnette et al., "Site-specific Mutagenesis of the Catalytic Subunit of Cholera Toxin: Substituting Lysine for Arginine 7 Causes Loss of Activity", page 273, abstract no. 53261, Infection and Immunity, 59(11), pages 4266-4270.	1-7, 10-16, 29-30, 33-36 8, 17-29, 31-32
Y	SCIENCE, Volume 232, issued 06 June 1986, C. Locht et al., "Pertussis Toxin Gene: Nucleotide Sequence and Genetic Organization", pages 1258-1264, especially page 1261-1262 paragraphs 1 and 6 and Fig. 3	1-33
Y	SCIENCE, Volume 242, issued 07 October 1988, W.N. Burnette et al., "Pertussis Toxin S1 Mutant With Reduced Enzyme Activity and a Conserved Protective Epitope", pages 72-74, entire document.	1-33